INTERNATIONAL APPLIC ON PUBLISHED UNDER THE PATENT OPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/566, 33/567, 33/53
C12Q 1/00

A1

(11) International Publication Number: WO 93/24838

(43) International Publication Date: 9 December 1993 (09.12.93)

(21) International Application Number: PCT/US93/04735

(22) International Filing Date: 21 May 1993 (21.05.93)

(30) Priority data:
891,366 29 May 1992 (29.05.92) US

(71) Applicant: ADEZA BIOMEDICAL CORPORATION [US/US]; 1240 Elko Drive, Sunnyvale, CA 94089 (US).

(72) Inventors: SENYEI, Andrew, E. ; 30551 Hilltop Way, San Juan Capistrano, CA 92675 (US). CASAL, David, C. ; 22441 Franklin Court, Mountain View, CA 94040 (US).

(74) Agents: TERLIZZI, Laura et al.; Skjerven, Morrill, MacPherson, Franklin & Friel, 25 Metro Drive, Suite 700, San Jose, CA 95110 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: SCREENING METHOD FOR IDENTIFYING WOMEN AT INCREASED RISK FOR PRETERM DELIVERY

(57) Abstract

The present invention provides an early, biochemical indication of increased risk of preterm delivery. The method comprises obtaining a body fluid sample from a pregnant patient after about week 4 of gestation and determining the proportion of total human chorionic gonadotropin (hCG) in the sample that is in the intact form. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

SCREENING METHOD FOR IDENTIFYING WOMEN AT INCREASED RISK FOR PRETERM DELIVERY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods for detection of increased risk of preterm delivery. In particular, this invention is directed to determining an early indication of increased risk of preterm delivery by determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form in a body fluid sample.

Description of the Prior Art

Determination of impending preterm births is critical for increasing neonatal survival of preterm infants. In particular, preterm neonates account for more than half, and maybe as much as three-quarters of the morbidity and mortality of newborns without congenital anomalies. Although tocolytic agents which can delay delivery were introduced 20 to 30 years ago, there has been only a minor decrease in the incidence of preterm delivery. It has been postulated that the failure to observe a larger reduction in the incidence of preterm births is due to errors in the diagnosis of preterm labor and to the patients' conditions being too advanced for tocolytic agents to successfully delay the birth.

Traditional methods of diagnosis of preterm labor have high false-negative and false-positive error rates [Friedman et al, *Am. J. Obstet. Gynecol.* **104**:544 (1969)]. In addition, traditional methods for determining impending preterm delivery, particularly in patients with clinically intact membranes, may require subjective interpretation, may require sophisticated training or equipment [Garl et al, *Obstet. Gynecol.*

60:297 (1982)] or may be invasive [Atlay et al., *Am. J. Obstet. Gynecol.* **108**:933 (1970)]. Accordingly, an early, objective biochemical marker indicative of increased risk for preterm delivery was sought.

Recently, Lockwood et al [*New Engl. J. Med.* **325**:669-674 (1991)] reported that fetal fibronectin in cervical and vaginal secretions indicates pregnancies which are at risk of imminent delivery. The authors postulate that damage to the fetal membranes may release fetal fibronectin into the cervix and vagina, thus giving rise to the biochemical marker.

Other markers which may be released in women with true threatened pregnancies can be used to screen those women who should be closely monitored and to provide additional information about the stage of the disease.

SUMMARY OF THE INVENTION

The present invention provides an early, biochemical indication of increased risk of preterm delivery. The method comprises obtaining a body fluid sample from a pregnant patient after about week 4 of gestation and determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form in the sample. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery. The test is preferably administered to women at about 4 weeks gestation and repeated at each prenatal visit (every two to four weeks) until at least week 37, preferably until delivery if the test is negative. For those patients whose assay result indicates an increased risk of preterm delivery, a test of the patient's fetal fibronectin level can be made to confirm the increased risk and to estimate how soon the delivery will be. In addition, those patients can be carefully monitored, as for other patients at risk.

The test is both a sensitive and specific screen for

pregnancies at risk and can detect an increased risk of preterm delivery as early as two to four weeks prior to delivery. The method not only allows early intervention in the course of preterm delivery but also provides an additional factor which can indicate those pregnancies at greatest risk.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a screening assay which provides an early, biochemical indication of increased risk of preterm delivery based on detection of a decreased proportion of intact human chorionic gonadotropin (hCG) in a body fluid sample. The method can provide an indication of impending delivery as early as two to three weeks prior to delivery. This method allows early intervention in the course of preterm delivery and provides an additional factor which can indicate those pregnancies at greatest risk.

The method comprises obtaining a body fluid sample, preferably serum or urine, from a pregnant patient after about week 4 of pregnancy and prior to about week 36 or 37, and determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates a patient who is at risk for preterm delivery. In a preferred embodiment, the proportion of hCG that is in the intact form is determined using an immunoassay. Since inflammatory conditions in the local membranes could damage trophoblast secreted proteins such as hCG, the amount of intact hCG in the local area as reflected in cervicovaginal secretion samples, as well as the amount systemically (e.g. in blood and urine samples) can be used as an indicator of the damage at the maternal fetal interface.

The present invention can determine increased risk of preterm delivery between weeks 4 and 37 of gestation.

Deliveries between 4 to 20 weeks gestation are generally termed spontaneous abortions rather than preterm deliveries. Term pregnancies are from 37 to 40 weeks.

Intact and Nicked Human Chorionic Gonadotropin (hCG)

The present invention is based on detection of a significant decrease in the proportion of intact hCG that occurs in body fluids of women who deliver preterm. Total hCG is the sum of nicked hCG and intact hCG. The proportion of intact hCG can be determined using any method that determines the relative amounts of any two of total hCG, nicked hCG, and intact hCG.

hCG is a glycoprotein hormone produced almost exclusively by the placenta. The polypeptide portion of hCG is a dimer that is composed of an alpha subunit (92 amino acid residues) and a beta subunit (145 amino acid residues), joined noncovalently. The beta chain contains a disulfide bridge between cysteines 38 and 57. The appearance of hCG in patient urine is currently the most commonly used indicator to determine pregnancy.

hCG is found in the blood and urine of pregnant women as a mixture of two forms: an intact form and a proteolytically nicked form. The nicked form is the same as the intact form but for a single break in the beta-subunit polypeptide chain between either residues 44 and 45, residues 47 and 48, or far less commonly, residues 46 and 47. The nicked form comprises, on average, about one quarter of the total hCG population in both the blood and urine of pregnant women who deliver at term.

The alpha- and beta-chains of hCG are also present as free subunits. In addition, a fragment of the beta-chain, called the beta-core fragment, which comprises beta-chain residues 6-40 disulfide-linked to beta-chain residues 55-92, may also be present.

Other known variants of hCG include truncated forms which lack the first two or three N-terminal amino acids of the alpha chain. However, these truncated forms are

characteristic of relatively rare pregnancy-associated cancers (hydatidiform mole and choriocarcinoma) and are not observed in normal pregnancies.

Patients to be Tested

The present method can be used on any pregnant woman following about 4 weeks gestation and prior to term (week 36 or 37). In addition to screening any pregnant woman to determine whether the patient is at risk for preterm delivery, the patients who are preferably screened are those patients with clinically intact membranes in a high risk category for preterm delivery, and more preferably, all those women whose pregnancies are not sufficiently advanced to ensure delivery of a healthy fetus. Ninety percent of the fetal morbidity and 100 percent of the fetal mortality associated with preterm delivery is for those fetuses delivered prior to 32 to 34 weeks gestation. Therefore, 32 to 34 weeks gestation is an important cutoff for the health of the fetus, and preferably women whose pregnancies are at least about 4 weeks and prior to 34 weeks in gestation are tested.

In addition there are a large number of factors known to be associated with the risk of preterm delivery. Those factors include multiple fetus gestations, incomplete cervix, uterine anomalies, polyhydramnios, nulliparity, previous preterm rupture of membranes or preterm labor, preeclampsia, first trimester vaginal bleeding, little or no antenatal care, and symptoms such as abdominal pain, low backache, passage of cervical mucus, and contractions. Any pregnant woman at 4 or more weeks gestation with clinically intact membranes and having one or more risk factors for preterm delivery is preferably tested throughout the risk period; i.e., until about week 34 to 37.

The Sample

The sample is a body fluid sample, preferably blood,

urine or cervicovaginal secretions, and is collected according to standard procedures. A blood sample can be a plasma or, preferably, a serum sample. The sample is preferably frozen following processing if the sample cannot be analyzed within a few hours of collection. The urine sample can be a random sample, preferably a first morning specimen, or more preferably, a 24 hour sample. Urine samples which are not assayed within 24 hours of collection are preferably stored at 4°C, and more preferably, are stored frozen. A cervicovaginal secretion sample is generally obtained from the vaginal cavity or the external cervical canal using a swab having an absorbent material; e.g., cotton or dacron.

Assay Procedure

As stated previously, the proportion of intact hCG can be determined using any method that determines the relative amounts of any two of total hCG, nicked hCG, and intact hCG. Immunoassays that quantitate total and intact hCG are preferred. However, non-quantitative assays that determine relative amounts of nicked and intact hCG or total and intact hCG in a given volume of sample can also be used.

Total hCG is the sum of the nicked and intact forms of hCG. The proportion of intact hCG is the amount of intact hCG divided by the amount of total hCG. Therefore, when the proportion of hCG that is nicked is known, the proportion that is intact is the difference of the total hCG minus the nicked proportion. Similarly, when the relative levels of nicked and intact hCG are known, the proportion of hCG that is intact is the level of intact hCG divided by the sum of the levels of the intact and nicked forms. Therefore, determining the proportion of nicked hCG is equivalent to determining the proportion of intact hCG. Preferably, the proportion of intact hCG is reported as a percentage.

The proportion of intact hCG is preferably determined

by quantitating intact hCG and total hCG using an immunoassay. Alternatively, assays that determine the relative amounts of proteins in a sample, such as Western blot assays, can be used to determine the relative amounts of intact hCG and nicked hCG to determine the proportion of intact hCG. Determining the proportion of total hCG that is in the intact form requires not only distinguishing nicked from intact hCG, but also distinguishing nicked and intact hCG from the free alpha- and free beta-chains.

Antibodies specific for hCG and its subunit chains are well known. At least two epitopes have been reported on the alpha-chain and at least three on the beta-chain. In addition to the epitopes on each of the chains, there is an epitope that is present on the intact hCG dimer which is not present on either of the free chains or on nicked hCG. See, for example, Krichevsky et al [*Endocrinology* **123**:584-593 (1988)] and the references cited therein, which describe various hCG epitopes. That article and the references cited therein are hereby incorporated by reference in their entireties.

Anti-hCG antibodies can be polyclonal or monoclonal for the purposes of the present invention and can be produced and purified by conventional methods. Such methods are described in a number of publications including Tijssen, P. Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theories of Enzyme Immunoassays New York: Elsevier (1985).

In addition, antibodies to hCG are available. For example, a polyclonal antibody specific for the beta-chain is commercially available (Bios Pacific, Inc., Emeryville, CA). Anti-alpha-chain antibodies are available from Unipath (Cambridge, U.K.). In addition, a monoclonal antibody, designated B109, that is specific for the hCG dimer [described in Krichevsky et al, *Endocrinology* **123**:584-593 (1988); Cole et al, *Endocrinology*

32:1559-1567 (1991)] is used at numerous centers in the U.S. to detect low levels of hCG. This antibody does not recognizes free alpha- or free beta-chains or nicked hCG and, therefore, can be used to distinguish intact hCG from nicked hCG.

At present, there is no known antibody specific for the nicked form of hCG. However, if an epitope is identified that is present on the nicked form that is not present on the intact form, the immunoassay can quantitate nicked hCG and total hCG or quantitate nicked hCG and intact hCG to determine the proportion of intact hCG in the sample.

A number of different types of immunoassays are well known using a variety of protocols and labels. The assay conditions and reagents may be any of a variety found in the prior art. The assay can be heterogeneous or homogeneous and is conveniently a sandwich assay.

The assay usually employs solid phase-affixed antibodies. The solid phase-affixed antibodies are combined with the sample. Binding between the antibodies and sample can be determined in a number of ways. Complex formation can be determined by use of soluble antibodies. The soluble antibodies can be labeled directly or can be detected using labeled second antibodies specific for the species of the soluble antibodies. Various labels include radionuclides, enzymes, fluorescers, colloidal metals or the like. Conveniently, the assay will be a quantitative enzyme-linked immunosorbent assay (ELISA) in which antibodies specific for hCG are used as the solid phase-affixed antibodies and enzyme-labeled, soluble antibodies.

To assay total hCG, the assay can use any two hCG antibodies for hCG that do not compete for a binding site, with the exception of dimer-specific antibodies. Preferably, the assay uses an antibody for the alpha-chain and an antibody for the beta-chain to avoid potential quantitation of either of the free chains. However, when

the relative amount of the free chains is not a significant proportion of total hCG, the assay can use two antibodies to the same chain. To assay intact hCG, the assay can use a dimer-specific antibody together with an antibody for the alpha-chain or, preferably, the beta-chain.

Alternatively, the assay can be based on competitive inhibition, where analyte in the sample competes with a known amount of analyte or analyte analog for a predetermined amount of anti-analyte antibody. In the competitive format, a dimer-specific antibody is used to detect intact hCG and an antibody for either the alpha-chain or the beta-chain is used to quantitate total hCG. When the relative amount of the free chains are not a significant proportion of the total hCG, the free chains do not affect the assay result. However, when the relative amounts of the free chains are significant, preferably an assay using an antibody for the alpha-chain and an antibody for the beta-chain such as a sandwich assay, rather than competitive assay, is used.

A standard with a known amount of intact hCG is used. Preferably, the hCG in the standard is at least 80% intact. Standards with known amounts of intact hCG are available commercially. As recently reported, the Columbia anti-hCG test that has been used to quantitate total hCG in some hCG standards does not detect nicked hCG [Birken et al, Endocrinology 129:1551-1558 (1991); Cole et al, *Endocrinology* 129:1559-1567 (1991)]. Therefore, the hCG standard is preferably one in which the proportion of intact hCG has been determined by amino acid sequence analysis.

Other quantitative methods to determine the proportion of hCG that is in the intact form can be envisaged. For example, a method to quantitate total and nicked hCG using amino acid sequence analysis of hCG purified from urine has been described [Khardana et al, *Endocrinology* 129:1541-1550 (1991)]. An exemplary

procedure to determine the proportion of hCG that is intact in a serum or urine sample by quantitating total and intact hCG is described in detail in the Examples.

In addition to methods that quantitate hCG forms, any method that determines relative amounts of total and intact hCG can also be used. For example, a Western blot assay can be used to determine the relative amounts of total and intact hCG by determining the relative amounts of nicked and intact hCG beta-chains. Briefly, the electrophoresis is performed under denaturing conditions which separate the alpha-chain from the beta-chain. A reducing agent, for example β-mercaptoethanol, is used to reduce disulfide bonds in the beta-chains. Following treatment with a reducing agent, the beta-chain of intact hCG remains a single chain with an apparent molecular weight of about 34 to 37 kD and the beta-chain of nicked hCG separates into an N-terminal fragment of about 17 kD and a C-terminal fragment of about 24 kD, as determined by electrophoresis.

Following electrophoresis, the separated proteins are transferred to a support membrane and detected with labeled anti-beta C-terminus antibody. The antibodies react with the intact beta-chain (34-37 kD) and the nicked beta-chain C-terminal fragment (24 kD). The size differences between the intact and nicked chains facilitate distinguishing the intact and nicked beta-chains. The relative amounts of intact and nicked beta-chain are determined by appropriate methods, depending on the label on the antibody. The proportion of hCG in the intact form is the relative amount of intact beta-chain divided by the sum of intact beta-chain and nicked beta-chain C-terminal fragment. Preferably, the original sample is also tested for free, intact beta-chain to ensure that the level of intact hCG is not overestimated.

A Western blot method to determine the proportion of hCG that is in the intact form is described in Cole et al

[*supra*]. Other methods which measure relative amounts of total, nicked, and intact hCG can also be envisaged.

Interpretation of Result

A decrease in the proportion of hCG that is in the intact form relative to the proportion that is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery. Preferably, the threshold value that separates risk from non-risk cases is two standard deviations below the average value for pregnancies that proceed to term. A preferred threshold below which the patient is considered to be at risk for preterm deliver is 25% or less intact hCG (as a fraction of total hCG in the sample). A more preferred value is 10% or less intact hCG. As is well known, the 25% threshold value will detect more false positive values. However, a somewhat high false positive rate is acceptable in a screening assay, where the objective is to detect all those at risk. For an assay which has a lower false positive rate, but which has a higher false negative rate, the lower threshold is selected. Since the proportion of intact hCG can also be expressed as the proportion of nicked hCG, or as the ratio of nicked to intact hCG, thresholds appropriate for such expressions can also be used. However, since the proportion of total hCG that is in the intact form varies considerably among women with pregnancies that proceed to term, it is preferable that patients with samples near the threshold value be retested in a follow up visit.

If the hCG test is positive (i.e., the proportion of hCG in the intact form is below the threshold value), the patient is preferably tested for the presence of fetal fibronectin in her cervicovaginal secretions. If fetal fibronectin is present in the secretions, the patient is likely to deliver in two to three days. Measures to determine or enhance fetal lung maturity can be undertaken. If the fetal fibronectin assay is negative,

the patient should be carefully monitored and repeated evaluations of the patient's fetal fibronectin levels should be performed on subsequent visits. In general, patients at risk for preterm delivery are examined every two weeks from about 22 to 36 weeks, rather than every four weeks as for patients in a low risk category.

If the hCG test is negative (the proportion of hCG in the intact form is above the threshold), the test is preferably repeated on each subsequent visit until either the test is positive or the patient reaches term.

The procedure is sensitive and specific. Since the test successfully detects a large percentage of patients who deliver early, the test is an effective screening procedure for women at risk for preterm delivery who do not have any other risk indicators.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Centigrade and concentrations as weight percent unless otherwise specified. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

EXAMPLE 1

The following immunoassay method determines the proportion of total hCG that is in the intact form. The method can be used for a serum or urine sample. The proportion of total hCG that is intact is determined by measuring the concentrations of intact hCG and total hCG by separate assays.

The concentration of intact hCG in the sample is determined using a B109:anti-β-peroxidase assay performed as described by Cole et al [*Endocrinology* 129:1559-1567 (1991)]. This immunoassay uses a hCG dimer-specific monoclonal antibody designated B109 (available from Drs. A. Krichevsky and E. Armstrong of Columbia University) as

the solid phase-affixed antibody to capture intact hCG, and peroxidase-labeled, goat anti-beta-chain antiserum (Bios Pacific, Inc., Emeryville, CA) to detect bound intact hCG. Microtiter plates are coated with antibody B109 (200 µl; 2 µg/ml in 0.25 M sodium carbonate, pH 9.5, containing 0.1 M NaCl) and plates are incubated overnight at 4°C. Plates are washed five times with water and aspirated before use.

In triplicate, sample or purified, intact hCG standard (100 µl) is added to coated wells together with buffer-carrier protein mix (100 µl; 0.05 M sodium phosphate, pH 7.5, containing 0.14 M NaCl and 0.1% ovalbumin). hCG standard solutions (0, 2.5, 5, 10, 15, 20, and 25 ng intact hCG/ml) are used to establish a standard curve.

The plates are shaken on a plate rotator overnight at ambient temperature and then washed five times with water and aspirated. Peroxidase-labeled goat anti-beta-chain antiserum (200 µl; 1:3500 dilution in 0.1 M Tris-HCl, pH 7.5, containing 0.025 M $CaCl_2$ and 0.1% ovalbumin) is then added to the wells, and the plates are shaken for 2 hours at ambient temperature. After another five washes with water, 200 µl of substrate mix is added (prepared by the addition of a 5 mg tablet of orthophenylenediamine [Sigma Chemical Company] and 4 µ l 30% H_2O_2 to 25 ml of 0.01 M sodium citrate, pH 4.9), and the plates are shaken in the dark for 30 min at ambient temperature. Hydrochloric acid (50 µl; 4 M) is added to stop the reactions, and the absorbance of the wells is determined in a TITERTEK MULTISCAN NCC-340 plate reader (Flow Laboratories, McClean, VA) at 492 nm. Data are sent to a Zenith 80286 computer, and standard curves are plotted and levels determined using TITERSOFT software (Flow Laboratories). All values are determined in triplicate. This assay is specific for intact hCG, with less than 1% cross-reactivity with free beta-subunit, free alpha-subunit, and human luteinizing hormone (hLH).

Total hCG is quantitated in the same way except that a monoclonal anti-alpha-chain antibody (Unipath) 4 μg/ml in the microtiter plate coating step) is used in place of the B109 antibody, a change that renders the assay equally specific for intact and nicked hCG. This assay is specific for total hCG, with less than 0.1% cross-reactivity with hCG free beta-subunit, free alpha-subunit, or hLH.

EXAMPLE 2

A serum sample from a pregnant women at 26 weeks gestation is assayed for intact and total hCG according to the procedure in Example 1. The proportion of hCG that is in the intact form in the sample is found to be 10%. This value indicates that the woman is at increased risk for preterm delivery.

EXAMPLE 3

A cervicovaginal secretion sample from a pregnant women at 26 weeks gestation is assayed for intact and total hCG according to the procedure in Example 1. The proportion of hCG that is in the intact form in the sample is found to be 75%. This value indicates that the woman is not at increased risk for preterm delivery.

WHAT IS CLAIMED IS:

1. A method for determining an early indication of increased risk of preterm delivery comprising
   a. obtaining a body fluid sample from a pregnant patient after week 4 and before week 37 of pregnancy; and
   b. determining the proportion of total human chorionic gonadotropin that is in the intact form in the sample, a decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicating an increased risk of preterm delivery.
2. The method of Claim 1 wherein the sample is a blood sample.
3. The method of Claim 2 wherein the sample is a serum sample.
4. The method of Claim 1 wherein the sample is a urine sample.
5. The method of Claim 1 wherein the proportion is determined by a non-quantitative assay.
6. The method of Claim 1 wherein the proportion is determined by a quantitative assay.
7. The method of Claim 6 wherein the level of total human chorionic gonadotropin and the level of intact human chorionic gonadotropin are quantitated using an immunoassay.
8. The method of Claim 7 wherein the immunoassay is a sandwich immunoassay.
9. The method of Claim 7 wherein the immunoassay is a competitive immunoassay.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/566, 33/567, 33/53; C12Q 1/00

US CL :436/501, 503, 510; 435/7.1, 7.92, 7.93, 7.94, 7.95

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/501, 503, 510; 435/7.1, 7.92, 7.93, 7.94, 7.95

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HENRY, "Clinical Diagnosis and Management by Laboratory Methods," published 1979 by W.B. Saunders Company (Philadelphia), see pages 680-692, see especially Figure 19 and pages 685-686.	1-9
Y	US, A, 4,310,455 (BAHL) 12 JANUARY 1982, see column 1, line 65 and column 12, line 42.	1-9
Y	US, A, 4,954,434 (MOROZ) 04 SEPTEMBER 1990, see column 5, line 31 and column 4, line 54.	1-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 June 1993	Date of mailing of the international search report 14 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DAVID R. PRESTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Endocrinology, Volume 129, No. 3, issued March 1991, L.A. Cole et al., "The hetrogeneity of human chorionic gonadotropin (hCG). III. The occurrence and biological and immunological activites of nicked hCG," pages 1559-1567, see especially page 1562, paragraph 1, right; page 1563, paragraph 2, left; page 1565, line 26, right; and page 1562, results section.	1-9

Hypoxia Alters Early Gestation Human Cytotrophoblast Differentiation/Invasion In Vitro and Models the Placental Defects that Occur in Preeclampsia

Olga Genbacev,* Rebecca Joslin,* Caroline H. Damsky,*‡ Bruno M. Polliotti,¶ and Susan J. Fisher*‡§‖

*Departments of *Stomatology, ‡Anatomy, §Pharmaceutical Chemistry, and ‖Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, San Francisco, California 94143-0512; and ¶Division of Infectious Diseases, Epidemiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30303*

Abstract

During normal human pregnancy a subpopulation of fetal cytotrophoblast stem cells differentiate and invade the uterus and its arterioles. In the pregnancy disease preeclampsia, cytotrophoblast differentiation is abnormal and invasion is shallow. Thus, the placenta is relatively hypoxic. We investigated whether lowering oxygen tension affects cytotrophoblast differentiation and invasion. Previously we showed that when early gestation cytotrophoblast stem cells are cultured under standard conditions (20% O_2) they differentiate/invade, replicating many aspects of the in vivo process. Specifically, the cells proliferate at a low rate and rapidly invade extracellular matrix (ECM) substrates, a phenomenon that requires switching their repertoire of integrin cell–ECM receptors, which are stage-specific antigens that mark specific transitions in the differentiation process. In this study we found that lowering oxygen tension to 2% did not change many of the cells' basic processes. However, there was a marked increase in their incorporation of [^{3}H]thymidine and 5-bromo-2′-deoxyuridine (BrdU). Moreover, they failed to invade ECM substrates, due at least in part to their inability to completely switch their integrin repertoire. These changes mimic many of the alterations in cytotrophoblast differentiation/invasion that occur in preeclampsia, suggesting that oxygen tension plays an important role in regulating these processes in vivo. (*J. Clin. Invest.* 1996. 97: 540–550.) Key words: trophoblast • preeclampsia • hypoxia • invasion • integrins

Introduction

Normal development of the placenta depends on differentiation of its specialized epithelial cells, termed trophoblasts (reviewed in 1, 2). Human cytotrophoblast stem cells follow one of two differentiation pathways. Those in floating villi detach from their basement membrane and fuse to form a syncytial layer that is in direct contact with maternal blood. These syncytiotrophoblasts play an important role in nutrient, waste, and gas exchange. In anchoring villi, cytotrophoblasts also fuse, but at specific locations many aggregate and form columns of mononuclear cells that attach to and then invade the decidualized endometrium and the first third of the myometrium (interstitial invasion), as well as the associated portions of uterine (spiral) arterioles (endovascular invasion). Cytotrophoblast invasion anchors the fetus to the mother and creates the large-diameter, low-resistance vessels that carry blood to the floating villi at the maternal–fetal interface.

We and others have used two approaches to study the differentiation process that results in human cytotrophoblast invasion of the uterus. First, analysis of tissue sections that contain both floating and anchoring villi allows descriptive studies of cytotrophoblast protein and RNA expression in vivo (3, 4). Second, we discovered that removing early gestation cytotrophoblast stem cells from their basement membranes and plating them on extracellular matrices (ECMs)[1] reprograms their fate such that they differentiate along the invasive pathway, rather than fuse to form syncytium (5–10). This in vitro model can be used to study mechanisms involved in the invasion process.

By using this combination of approaches, we showed that human cytotrophoblast invasion is accompanied by a reduction in the cells' proliferative capacity and requires the regulated expression of either proteinases or adhesion molecules. As they acquire an invasive phenotype, cytotrophoblasts produce and activate the 92-kD type IV collagenase, matrix metalloproteinase 9 (5). They also switch their repertoire of integrin cell–ECM adhesion receptors (3). In the first trimester human placenta, α6 integrins (laminin receptors) are localized primarily to cytotrophoblast stem cells, and their expression is downregulated during invasion. In contrast, the expression of integrins α5β1 (fibronectin receptor) and α1β1 (receptor for collagens I and IV and laminin) is sequentially upregulated in differentiating and invading cytotrophoblasts. By using reagents that specifically inhibit either matrix metalloproteinase-9 or cytotrophoblast integrin functions, we showed that matrix metalloproteinase-9 and α1β1 promote invasion in vitro, whereas α5β1 integrin inhibits this process (6, 7, 9). Thus, a balance between forces that promote or inhibit invasion is critical to placentation.

We have been interested in diseases of pregnancy that are associated with abnormal placentation as a means of understanding the in vivo relevance of our in vitro function perturbation experiments. Preeclampsia, which affects 7–10% of all first-time pregnancies (reviewed in reference 11), is one such syndrome. Compared with normal pregnancy, cytotrophoblasts in preeclampsia show an increased ability to proliferate

Address correspondence to Susan Fisher, Department of Stomatology, HSW 604, 513 Parnassus Avenue, University of California, San Francisco, CA 94143-0512. Phone: 415-476-5297; Fax: 415-476-4204; E-mail: sfisher@cgl.ucsf.edu

Received for publication 18 April 1995 and accepted in revised form 29 September 1995.

J. Clin. Invest.

0021-9738/96/01/540/11 $2.00
Volume 97, Number 2, January 1996, 540–550

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; ECM, extracellular matrix; hCG, human chorionic gonadotropin; hPL, human placental lactogen.

Contents

The Journal of Clinical Investigation
January 15, 1996, Volume 97, Number 2

Editorial

Probing the depths of degradation: matrix metalloproteinase-2 and endometrial menstrual breakdown. *L. A. Liotta* 273

Perspectives

Biological insights through genomics: mouse to man *E. M. Rubin and G. S. Barsh* 275

■ Molecular Medicine / Genetic Disorders

A nonsense mutation in the erythrocyte band 3 gene associated with decreased mRNA accumulation in a kindred with dominant hereditary spherocytosis. *P. B. Jenkins, G. K. Abou-Alfa, D. Dhermy, E. Bursaux, C. Féo, A. L. Scarpa, S. E. Lux, M. Garbarz, B. G. Forget, and P. G. Gallagher* 373

Regulation of transforming growth factor-β1 expression by the hepatitis B virus (HBV) X transactivator. *Y. D. Yoo, H. Ueda, K. Park, K. C. Flanders, Y. I. Lee, G. Jay, and S.-J. Kim* 388

Bone mineralization in an osteogenesis imperfecta mouse model studied by small-angle x-ray scattering. *P. Fratzl, O. Paris, K. Klaushofer, and W. J. Landis* 396

Antisense oligodeoxynucleotides selectively suppress expression of the mutant α2(I) collagen allele in type IV osteogenesis imperfecta fibroblasts. A molecular approach to therapeutics of dominant negative disorders. *Q. Wang and J. C. Marini* 448

In vivo trafficking of adoptively transferred interleukin-2 expanded tumor-infiltrating lymphocytes and peripheral blood lymphocytes. Results of a double gene marking trial. *J. S. Economou, A. S. Belldegrun, J. Glaspy, E. M. Toloza, R. Figlin, J. Hobbs, N. Meldon, R. Kaboo, C.-L. Tso, A. Miller, R. Lau, W. McBride, and R. C. Moen* 515

Effects of renin gene transfer on blood pressure and renin gene expression in a congenic strain of dahl salt-resistant rats. *E. M. St. Lezin, M. Pravenec, A. L. Wong, W. Liu, N. Wang, S. Lu, H. J. Jacob, R. J. Roman, D. E. Stec, J.-M. Wang, I. A. Reid, and T. W. Kurtz* 522

Mapping a cardiomyopathy locus to chromosome 3p22-p25. *T. M. Olson and M. T. Keating* 528

Cardiac-specific overexpression of phospholamban alters calcium kinetics and resultant cardiomyocyte mechanics in transgenic mice. *V. J. Kadambi, S. Ponniah, J. M. Harrer, B. D. Hoit, G. W. Dorn II, R. A. Walsh, and E. G. Kranias* 533

■ Infection / Inflammation / Immunity

Recombinant interferon-alpha selectively inhibits the production of interleukin-5 by human CD4+ T cells. *L. Schandené, GF. F. Del Prete, E. Cogan, P. Stordeur, A. Crusiaux, B. Kennes, S. Romagnani, and M. Goldman* 309

Functional expression of fas and fas ligand on human gut lamina propria T lymphocytes. A potential role for the acidic sphingomyelinase pathway in normal immunoregulation. *R. De Maria, M. Boirivant, M. G. Cifone, P. Roncaioli, M. Hahne, J. Tschopp, F. Pallone, A. Santoni, and R. Testi* 316

Dynamics of viral replication in infants with vertically acquired human immunodeficiency virus type 1 infection. *A. De Rossi, S. Masiero, C. Giaquinto, E. Ruga, M. Comar, M. Giacca, and L. Chieco-Bianchi* 323

Antigen targeting to myeloid-specific human FcγRI/CD64 triggers enhanced antibody responses in transgenic mice. *I. A. F. M. Heijnen, M. J. van Vugt, N. A. Fanger, R. F. Graziano, T. P. M. de Wit, F. M. A. Hofhuis, P. M. Guyre, P. J. A. Capel, J. S. Verbeek, and J. G. J. van de Winkel* 331

Modifications of myelin basic protein in DM20 transgenic mice are similar to those in myelin basic protein from multiple sclerosis. *F. G. Mastronardi, B. Mak, C. A. Ackerley, B. I. Roots, and M. A. Moscarello* 349

A novel *escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J. E. Somerville, Jr., L. Cassiano, B. Bainbridge, M. D. Cunningham, and R. P. Darveau* 359

In vitro and in vivo effects of leukotriene B_4 antagonism in a primate model of asthma. *C. R. Turner, R. Breslow, M. J. Conklyn, C. J. Andresen, D. K. Patterson, A. Lopez-Anaya, B. Owens, P. Lee, J. W. Watson, and H. J. Showell* 381

Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor κB-dependent transcriptional activation. *Z. Zhu, W. Tang, A. Ray, Y. Wu, O. Einarsson, M. L. Landry, J. Gwaltney, Jr., and J. A. Elias* 421

IgA class switch in Iα exon–deficient mice. Role of germline transcription in class switch recombination. *G. R. Harriman, A. Bradley, S. Das, P. Rogers-Fani, and A. C. Davis* 477

Murine Vλx and Vλx-containing antibodies bind human myelin basic protein. *F. S. Galin, C. C. Maier, S.-R. Zhou, J. N. Whitaker, and J. E. Blalock* 486

Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. *D. J. Pinsky, Y. Naka, H. Liao, M. C. Oz, D. D. Wagner, T. N. Mayadas, R. C. Johnson, R. O. Hynes, M. Heath, C. A. Lawson, and D. M. Stern* 493

Imogen 38: A novel 38-kD islet mitochondrial autoantigen recognized by T cells from a newly diagnosed Type 1 diabetic patient. *S. D. Arden, B. O. Roep, P. I. Neophytou, E. F. Usac, G. Duinkerken, R. R. P. de Vries, and J. C. Hutton* 551

* Purification and characterization of a naturally processed Hepatitis B virus peptide recognized by CD8+ cytotoxic T lymphocytes. *S.-L. Tsai, M.-H. Chen, C.-T. Yeh, C.-M. Chu, A.-N. Lin, F.-H. Chiou, T.-H. Chang, and Y.-F. Liaw* 577

■ Hormones / Cytokines/ Signaling

Energy-ubiquitin–dependent muscle proteolysis during sepsis in rats is regulated by glucocorticoids. *G. Tiao, J. Fagan, V. Roegner, M. Lieberman, J.-J. Wang, J. E. Fischer, and P.-O. Hasselgren* 339

Endothelin-3 induces hypertrophy of cardiomyocytes by the endogenous endothelin-1–mediated mechanism. *M. Tamamori, H. Ito, S. Adachi, H. Akimoto, F. Marumo, and M. Hiroe* 366

Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium. *M. L. Iruela-Arispe, P. Porter, P. Bornstein, and E. H. Sage* 403

Detection of receptors for interleukin-6, interleukin-11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in bone marrow stromal/osteoblastic cells. *T. Bellido, N. Stahl, T. J. Farruggella, V. Borba, G. D. Yancopoulos, and S. C. Manolagas* 431

Human endometrial matrix metalloproteinase-2, a putative menstrual proteinase. Hormonal regulation in cultured stromal cells and messenger RNA expression during the menstrual cycle. *J. C. Irwin, D. Kirk, R. B. L. Gwatkin, M. Navre, P. Cannon, and L. C. Giudice* 438

(continued on the reverse side of this cover)

(12). In addition, invasion is shallow and fewer arterioles are breached, an important factor with regard to the reduction in uteroplacental blood flow observed in this syndrome (13). How abnormal placentation translates into the clinical presentation of the disease, which includes fetal intrauterine growth retardation and maternal hypertension and proteinuria, is not yet clear (11, 14). However, work from our laboratory showed that lack of invasion is associated with specific alterations in the cytotrophoblasts' ability to differentiate. They express integrin $\alpha5\beta1$ but not $\alpha1\beta1$ (15, 16), an adhesive phenotype which our in vitro experiments predict would restrain invasion.

Together, these results suggest that oxygen tension could regulate the cytotrophoblasts' ability to differentiate and, as a consequence, to express proteins that are critical to the invasion process. In mammalian cells that have been studied in this context (e.g., endothelial cells, tumor spheroids) hypoxia upregulates the expression of stress (oxygen-regulated proteins), heat shock, and glucose-regulated proteins, as well as cytokines and growth factors (17–21). These include erythropoietin (22, 23), PDGF B chain (24), endothelin (25), IL-1α (26), IL-8 (27), ornithine decarboxylase (28), vascular endothelial growth factor (29), glyceraldehyde-3-phosphate dehydrogenase (30), xanthine dehydrogenase/xanthine oxidase (31), and metallothionein IIA (32).

The *cis*-acting DNA sequences and the *trans*-acting transcription factors that regulate the expression of these hypoxia-induced proteins are beginning to be elucidated. For example, hypoxia-inducible factor 1, a nuclear protein whose DNA-binding activity is induced in hypoxia, upregulates erythropoietin production by binding to an enhancer element (5′-TACGTGCT-3′) in the 3′ region of this gene (33, 34). Hypoxia-inducible factor 1 also induces transcription of glycolytic enzymes, which are important to the cells' ability to switch from aerobic respiration to glycolysis for energy production (35). The expression and/or activity of several other transcription factors, including Fos and Jun (components of the AP-1 complex; 36), nuclear factor κB (37), and the heat shock transcription factor (38), also increase in hypoxia.

These studies suggest that hypoxia can specifically alter a cell's gene expression and protein repertoire. Here we report the results of experiments in which we analyzed the effects of varying the oxygen content of the culture atmosphere on differentiation/invasion of early gestation cytotrophoblasts in vitro. The impetus for these experiments was the dramatic changes in oxygen content of the placental environment that normally occur during early gestation. Namely, before the tenth week of pregnancy there is little blood flow to the intervillous space, due to the fact that cytotrophoblast invasion is largely interstitial, rather than endovascular. As a result, the placenta proper is relatively hypoxic. Rodesch et al. (39) used a polarographic oxygen electrode to show that the mean oxygen pressure of the intervillous space is 17.9±(SD) 6.9 mmHg at 8–10 wk gestation as compared with 39.6±12.3 in the endometrium. By 12–13 wk the oxygen pressure in the intervillous space is 60.7±8.5 mmHg, a reflection of the fact that cytotrophoblasts are beginning to substantially remodel the spiral arterioles. Consequently, blood flow to the intervillous space increases dramatically. Presumably the most invasive cytotrophoblasts, e.g., those that line these arterioles, are exposed to oxygen concentrations similar to those of arterial blood (95–100 mmHg).

Preeclampsia is associated with failure of cytotrophoblasts to invade the spiral arterioles (15). We hypothesized that in this disease the events that normally take place during the first trimester of pregnancy, which convert the maternal–fetal interface from a relatively hypoxic environment to one that is relatively well oxygenated, fail to occur. Consequently, cytotrophoblast stem cells remain in a relatively hypoxic environment. We were interested in determining whether this could affect the cells' ability to differentiate, a prerequisite for invasion. Our findings suggest this is the case. Specifically, we found that hypoxia affects progression of cytotrophoblasts through the cell cycle. In addition, their invasiveness is greatly reduced due at least in part to alterations in integrin cell–ECM receptor expression. Together, these results suggest that hypoxia alters the balance between proliferation and differentiation of cytotrophoblasts and initiates a feedback mechanism that inhibits their differentiation and invasion, thus setting the stage for later pregnancy complications such as preeclampsia.

Methods

Cytotrophoblast isolation and culture. Placentas were obtained immediately after first (10–12 wk) and second (16–24 wk) trimester terminations. Cytotrophoblasts were isolated from pools of multiple placentas by published methods (6). In all cases, remaining leukocytes were removed by using an antibody to CD-45, a protein tyrosine phosphatase found on bone marrow–derived cells (40), coupled to magnetic beads. Previous work from this laboratory showed that this method removes contaminating nontrophoblast cells, as demonstrated by the absence of classical HLA class I antigens (41). The resulting cells were plated on Matrigel-coated substrates (Collaborative Biomedical Products, Bedford, MA) and cultured in DME H21 MEM containing 2% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 50 μg/ml gentamicin (6).

Cytotrophoblasts were either maintained under standard tissue culture conditions (5% CO_2/95% air) or placed in a Bactron anaerobic incubator (Sheldon Manufacturing Inc., Cornelius, OR) where they were maintained in a 2% O_2/93% N_2/5% CO_2 environment. The incubator was located inside a chamber that allowed manipulation of the cells in the same atmosphere. In experiments in which we used three different concentrations of oxygen (2, 8, and 20%), cells maintained in 8% oxygen were cultured in a Modular incubator chamber (Billups-Rothenberg, Del Mar, CA). The chamber was filled with a mixture of O_2, N_2, and CO_2 to achieve a final oxygen concentration of 8% and a final CO_2 concentration of 5%. Dissolved O_2 at the cell-medium interface, measured using microoxygen electrodes (MI-730; Microelectrode Inc., Londonderry, NH), was 20% (98 mmHg) under standard tissue culture conditions, 8% in the Modular incubator chamber (56 mmHg), and 2% (14 mmHg) in the Bactron anaerobic incubator.

Assaying the effects of hypoxia on cytotrophoblast protein synthesis, glucose metabolism, and hormone secretion. Cytotrophoblasts (10^6) were plated in 16-mm Matrigel-coated tissue culture wells (6). Routinely, the cells were maintained in either 2 or 20% oxygen. When indicated, the cells were also incubated in an 8% oxygen atmosphere.

Incorporation of [^{35}S]methionine/cysteine into cellular and secreted proteins was quantified as follows. Unless otherwise indicated, all reagents were obtained from Sigma Chemical Co., St. Louis, MO. Cytotrophoblasts were cultured for 12 h on Matrigel substrates. Then they were washed three times with methionine-free DME, after which they were cultured for an additional 12 h in 1 ml of the same medium containing 100 μCi of a mixture of [^{35}S]methionine and [^{35}S]cysteine (1175 Ci/mmol; New England Nuclear; Wilmington, DE). At the end of the labeling period the cells were washed twice with 1 ml fresh DME, then detached from the Matrigel and dispersed

by treatment with Dispase (0.5 ml/well; Collaborative Biomedical Products). After 2 h at 37°C the enzymatic activity was stopped by adding 1 ml of 5 mM EDTA. Cytotrophoblasts were washed three times by centrifugation (800 *g*, 5 min), resuspended in 1 ml Ca^{2+}-, Mg^{2+}-free PBS, and the number of cells was counted in a hemocytometer. Then the cytotrophoblasts were again pelleted by centrifugation and resuspended (10^6 cells/100 μl) in lysis buffer (PBS containing 1% Triton X-100, 1 mM PMSF, 5 μg/ml DNase I, 5 μg/ml RNase A, 5 mM $MgCl_2$, 5 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 μg/ml soybean trypsin inhibitor). Aliquots (3 × 100 μl) of cell lysate were precipitated with 100 μl of ice-cold 20% TCA for 20 min at 4°C. The resulting suspensions were collected by vacuum filtration onto glass microfiber disks. The disks were washed twice with 5 ml ice-cold 10% TCA and once with 5 ml 100% ethanol, then transferred to vials containing scintillation cocktail (Ultima-gold; Packard Instruments, Meriden, CT), and radioactivity was determined using a LS1701 counter (Beckman Instruments Inc., Brea, CA). The conditioned medium (100 μl) was frozen until it was subjected to TCA precipitation in parallel with the cell pellet. Cells and medium from three replicate cultures established from each of five separate cell isolation procedures were analyzed.

The amount of glucose and lactate in conditioned medium from cells cultured for 72 h was determined enzymatically using commercial glucose oxidase and lactate dehydrogenase assay kits (Sigma). Values were calculated as the difference between glucose and lactate in conditioned medium and fresh culture medium. Culture medium from three replicate cultures established from each of 10 separate cell isolation procedures was analyzed.

Cytotrophoblast secretion of human chorionic gonadotropin (hCG) and human placental lactogen (hPL) during the first 72 h of culture was measured by competition ELISA. Wells of ELISA plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with antigen by adding 100 μl of a stock solution of either hPL (bicarbonate buffer, pH 9.5) or βhCG (1 μg/ml PBS) and incubating overnight at 4°C. Both hormones were obtained from Sigma. Routinely, 75 μl of medium was transferred to cluster tubes, and then an equal volume of antibodies diluted in PBS containing 0.05% Tween-20 and 1% nonfat dried milk (PBST) was added. The mouse monoclonal anti-hPL antibody (INN hPL5, Harlan Bioproducts for Science, Indianapolis, IN) was used at a dilution of 1:13,000 (vol/vol) and the rabbit polyclonal anti-βhCG antibody (Hycor Biomedical Inc., Irvine, CA) at a dilution of 1:10,000 (vol/vol). Then the samples were incubated at 37°C for 1 h. Just before the start of the assay, the ELISA plates were blocked by incubating for at least 45 min in 100 μl PBST. Then the wells were rinsed once with PBST and emptied, after which 100 μl of the medium–antibody mixture was transferred to each well. The samples were incubated for 2 h and washed five times with PBST. Then 100 μl of a solution of peroxidase-conjugated secondary antibody (goat anti–mouse for hPL, 1:1000 [vol/vol] in PBST; goat anti–rabbit for βhCG, 1:3000 [vol/vol] in PBST; both obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added and the samples were incubated for an additional 1 h. After extensive washing with PBST, 100 μl of a 5 mg/ml solution of *o*-phenylenediamine substrate in 85 mM citrate buffer was added to each well. The samples were incubated in the dark for 10 min to allow color development. The reaction was stopped by adding 50 μl 4 N H_2SO_4 to each well. A_{490} was determined, and data were generated by four-parameter curve analysis using a reference preparation of either hPL (World Health Organization [WHO], NIBSC 75/545) or βhCG (WHO, 1st IRP 75/551). The standard curves covered six dilutions; 3–100 ng for hPL and 5–160 mIU/ml for βhCG. Culture medium from three replicate cultures established from each of five separate cell isolation procedures was analyzed.

Assaying the effects of hypoxia on cytotrophoblast DNA synthesis. DNA synthesis was assayed in two ways. First, cytotrophoblasts (10^6) from 10–12 wk placentas were plated in 16-mm Matrigel-coated tissue culture wells (6) in either a 2 or a 20% oxygen atmosphere. After 48 h, medium containing 5 μCi/ml [^{3}H]thymidine (44 Ci/mM; Amersham Corp., Arlington Heights, IL) was added. After 72 h the medium was aspirated. The cells were washed three times with 2 ml PBS containing unlabeled thymidine (5 μg/ml), treated with Dispase, and lysed. Radioactivity was measured after TCA precipitation as described above. Cells and medium from three replicate cultures prepared from each of three separate isolation procedures were analyzed.

Second, cytotrophoblasts (2.5 × 10^5) isolated from two separate pools of 10–12 wk gestation placentas were cultured on Matrigel-coated filters for 48 h in either a 2, an 8, or a 20% oxygen environment. The culture medium was aspirated, and medium containing 1 μM of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU; Sigma Chemical Co.), was added. After 24 h the experiment was terminated. The filters were washed 2× for 20 min in PBS, fixed in 3% paraformaldehyde for 1 h at 4°C, washed again in PBS (3×, 10 min), and permeabilized in cold methanol for 10 min. After rinsing in PBS (3×, 20 min) they were incubated with a fluorescein-labeled anti-BrdU antibody according to the manufacturer's directions (Boehringer Mannheim Biochemicals). To verify that cells were cytotrophoblasts, the filters were rinsed again in PBS (3×, 10 min), incubated overnight with a rat anticytokeratin mAb (7D3; 3), and immunoreactivity was detected using a goat anti–rat IgG mAb conjugated to Texas red (Jackson ImmunoResearch Laboratories Inc.). Filters were dissected from the inserts with a scalpel blade and mounted on polylysine-coated slides in a drop of mounting medium such that the top of the filter faced upward. The percentage of cytokeratin-positive cells that incorporated BrdU was determined by examining 500 cells from five randomly selected areas of each filter. Two filters were analyzed from each experimental group.

Invasion assays. Two assays were used. First, cytotrophoblasts were cultured with decidual explants. Briefly, portions of the decidua parietalis, which does not contain cytotrophoblasts before culture, were obtained at the time of pregnancy termination (10–12 wk). The tissue was washed three times in ice-cold PBS and cut into 2-mm^3 pieces. After washing another three times, five decidual pieces in 500 μl medium (DME /2% Nutridoma) were plated on each of several Millicel inserts (12-mm diameter; Millipore Corp., Bedford, MA) coated with 200 μl Matrigel. The inserts were transferred to 16-mm culture dishes containing 600 μl medium and incubated for 6 h, the amount of time required for the explants to attach to the ECM substrate. Then the medium was aspirated from the upper chamber, and 5 × 10^5 cytotrophoblasts in 500 μl of medium was added. The cultures were maintained for up to 4 d, and medium was changed daily. At various time points (24, 48, 72, and 96 h) the samples were fixed in 3% paraformaldehyde for 40 min, washed three times in PBS (4°C, 10 min), infiltrated with sucrose, embedded in OCT (Miles Scientific, Naperville, IL), and frozen in liquid nitrogen. Sections (6 μm) were cut using a cryostat (Slee International Inc., Tiverton, RI) and collected on poly-L-lysine–coated microscope slides. The sections were incubated for 1 h with primary anticytokeratin antibody (7D3), which reacts with cytotrophoblasts but not other villous components, diluted 1:100 (vol/vol) in PBS. Then they were rinsed in PBS (three times for 10 min) and incubated for 30 min with rhodamine-conjugated goat anti–rat IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 (vol/vol) in PBS. The samples were examined using a confocal microscope (600; Bio-Rad Laboratories, Richmond, CA). Three explants from each of five separate isolation procedures were analyzed.

Second, to quantify invasion, cytotrophoblasts (2 × 10^5) were cultured in Matrigel-coated Transwell inserts (6.5 mm; Costar Corp.) containing polycarbonate filters with 8-μm pores (6) in either a 2 or a 20% oxygen atmosphere. After 72 h the cultures were rinsed in PBS, fixed, and stained with anticytokeratin antibodies as described above. To assay invasion the filters were dissected from the inserts with a scalpel blade and mounted on poly-L-lysine–coated slides in a drop of mounting medium such that the underside of the filters faced upward. Serial optical sections (1 μm) that included the upper (Matrigel-coated) and lower filter surfaces, as well as the pores that connected

them, were obtained by using confocal microscopy. The number of cytokeratin-positive cell processes that penetrated the Matrigel and appeared on the underside of the filters was counted. Three filters were used for each experimental group. The total number of filters analyzed for cells in each gestational age group is summarized in Fig. 4. In additional experiments cytotrophoblasts were cultured in a 2, an 8, and a 20% oxygen atmosphere. The total number of filters analyzed in each experimental group is summarized in Fig. 5.

Statistical analyses. Statistical significance of the data was tested using Student's paired *t* test. Results were considered significant at the 0.05 level.

Immunofluorescence localization. Tissue samples of the normal and preeclamptic placental bed were obtained as previously described (3, 15). The methods we used to fix the samples and to detect cytokeratin-positive cytotrophoblasts have also been described (3, 15).

Integrin and ECM expression by cultured cytotrophoblasts was analyzed by confocal microscopy. The cells were fixed and processed as described above. In all cases the cells were double stained with either antiintegrin ($\alpha 5$, $\alpha 1$, $\beta 1$) or anti-ECM (collagen IV and fibronectin) antibodies and antibodies that detect cytokeratin as described previously (3, 5, 9). The samples were analyzed by using dual channel fluorescence imaging.

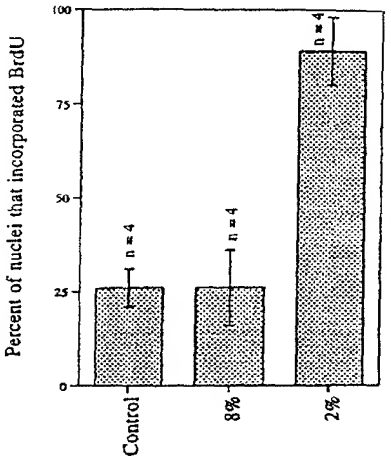

Figure 1. Culturing cytotrophoblasts in a 2% oxygen atmosphere produced a threefold increase in their incorporation of BrdU compared with control cells cultured in 2% oxygen ($P < 0.01$). Cells were isolated from two separate pools of 10–12 wk gestation placentas. The percentage of cytokeratin-positive cells that incorporated BrdU was determined by examining 500 cells from five randomly selected areas of each filter. Two filters were established from two different isolation procedures. Error bars show SD.

Results

Many cytotrophoblast cellular processes were not affected by hypoxia. Initial experiments suggested, based on the cell's morphology, that culturing cytotrophoblasts for 72 h in 2% O_2 (comparable to the oxygen concentration at the intervillous space at 8–10 wk gestation) had no obvious negative effects. To confirm this impression we assayed the cells' ability to exclude Trypan blue. In a 20% O_2 atmosphere 93.5±3.8% of all the cells (attached and unattached; see below) excluded this dye, as compared with 93.5±6.4% of the cells cultured in a 2% O_2 atmosphere.

We also assayed several parameters that reflected both generalized and specialized aspects of the cells' functions (Table I). As to the general state of the cells, those cultured in 20 or 2% oxygen incorporated nearly identical amounts of [^{35}S]methionine/cysteine into proteins. Likewise, they secreted comparable amounts of TCA-precipitable, ^{35}S-labeled proteins into the culture medium. Recent experiments in which we analyzed, by using 2-D gel analyses, the effects of hypoxia on cytotrophoblast production of individual ^{14}C-labeled proteins substantiated these results (data not shown). Of the ~200 spots seen on the gels, the intensity of only 10 changed (7 upregulated, 3 downregulated [Falk, R., and S. J. Fisher, manuscript in preparation]). Similarly, there were no significant differences in either glucose uptake or lactate production by cells maintained under the two culture conditions. As to specialized cytotrophoblast functions, we assayed the effects of hypoxia on hormone release. Secretion of neither human chorionic gonadotropin nor human placental lactogen changed in response to lowering oxygen tension.

Hypoxia stimulates cytotrophoblast incorporation of [^{3}H]thymidine and BrdU. In many cells, differentiation is coupled with exit from the cell cycle. Cytotrophoblast stem cells and those that are in the initial stages of differentiation in vivo react with antibodies that recognize Ki67 and proliferating cell nuclear antigen, antigens expressed by dividing cells (42, 43).

Table I. The Effects of Hypoxia on Cytotrophoblast Protein Synthesis, Glucose Metabolism, Hormone Secretion, and [^{3}H]thymidine Incorporation (values are means ±SE)

Parameter assayed	Oxygen content of medium	
	20%	2%
1 × 10^6 cells		
^{35}S-amino acid incorporation into TCA-precipitable protein (cpm)*		
Medium (n = 15)	0.3 × 10^6±0.4 × 10^5	0.3 × 10^6±0.3 × 10^5
Cell-associated (n = 15)	1.6 × 10^6±0.3 × 10^5	1.4 × 10^6±0.2 × 10^5
Glucose consumption (μg)‡ (n = 30)	2.6±0.4	3.1±0.5
Lactate production (mg)‡ (n = 30)	5.2±7	4.8±9
hCG secretion (mIU)‡ (n = 15)	60±4.8	58±5.3
hPL secretion (ng)‡ (n = 15)	638±172	585±168
[^{3}H]thymidine incorporation (cpm)§ (n = 9)	8,569±2,890	18,689±1,479‖

*Samples were collected after cytotrophoblasts were cultured for 12 h. ‡Conditioned medium was collected after cytotrophoblasts were cultured for 72 h. §Cytotrophoblasts were incubated with [^{3}H]thymidine-containing media between 48 and 72 h of culture. ‖$P < 0.05$.

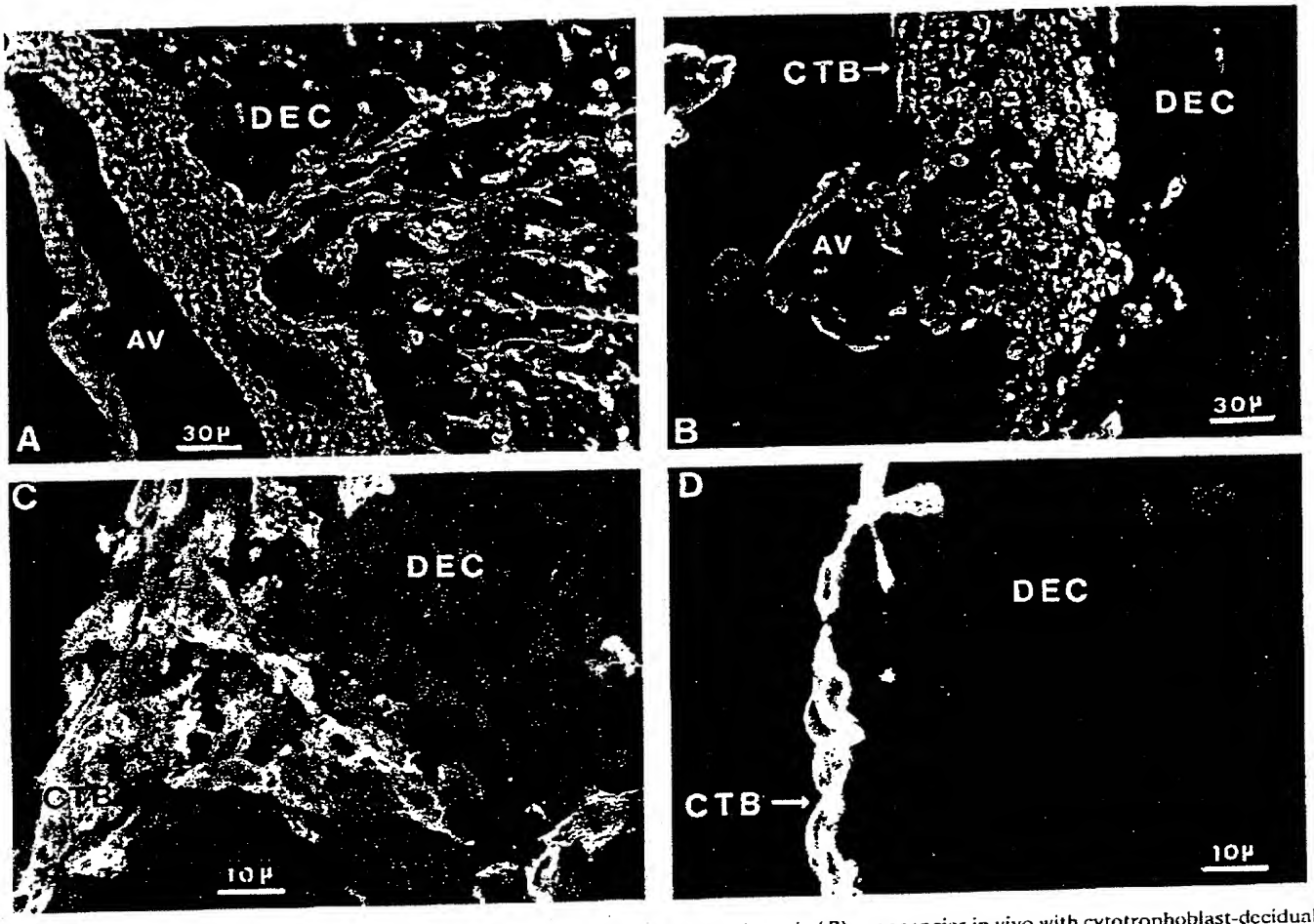

Figure 2. Comparing human cytotrophoblast invasion in normal (*A*) and preeclamptic (*B*) pregnancies in vivo with cytotrophoblast-decidual interactions in cultured explants maintained in 20% (*C*) or 2% (*D*) oxygen. Frozen sections of the maternal–fetal interface (36 wk) were stained with anticytokeratin antibodies and fluorescein-conjugated secondary antibodies. In normal pregnancy (*A*), cytokeratin-positive cytotrophoblasts (*CTB*) connected anchoring villi (*AV*) to the uterus and were found throughout the decidua (*DEC*). In preeclampsia (*B*), cytotrophoblast invasion was limited to the superficial portion of the decidua. We compared these interactions with those occurring when purified first trimester human cytotrophoblasts invaded decidual explants in vitro. After 72 h the cultures were fixed, stained with anticytokeratin antibodies and fluorescein-conjugated secondary antibodies, and then viewed by confocal microscopy. In a 20% oxygen atmosphere (*C*), cytokeratin-positive cytotrophoblasts attached to and invaded the decidua. In a 2% oxygen atmosphere (*D*), cytotrophoblasts attached to the decidual explants, but invasion was significantly reduced.

but those which are fully differentiated do not. To begin to understand whether hypoxia affects cytotrophoblast differentiation, we determined whether lowering the oxygen tension changes the fraction of cytotrophoblasts that enter S phase. We found that cells cultured in 2% oxygen incorporated twice the amount of [^{3}H]thymidine as did those cultured in a 20% oxygen atmosphere (Table I).

Similarly, BrdU incorporation increased approximately threefold when 10–12 wk human cytotrophoblasts were cultured in a 2%, as compared with a 20%, oxygen environment (Fig. 1). We also studied BrdU incorporation by cytotrophoblasts that were incubated in an 8% oxygen atmosphere, i.e., comparable to the oxygen content measured in the endometrium during the first trimester and in the intervillous space after 12 wk of pregnancy (39). Incorporation was virtually identical to that observed under standard tissue culture conditions (20% O_2).

Hypoxia inhibits cytotrophoblast invasion. Next, we determined whether hypoxia inhibits cytotrophoblast differentiation along the invasive pathway. We assayed two endpoints: invasive behavior (described in this section) and integrin, stage-specific antigen expression (described in the next section). In vivo, differentiation/invasion of cytotrophoblast stem cells results in penetration of the uterus and its blood vessels (3). In vitro the cells invade decidual explants or isolated ECMs (5, 6). Here we used both of these invasion models. In 20% oxygen, interactions between cytotrophoblasts and decidual explants appeared similar to those of cytotrophoblasts invading the decidua in vivo (compare Figs. 2, *A* and *C*); elongated, cytokeratin-positive cells invaded deeply into the uterine tissue. In 2% oxygen, there was no change in the number of cytotrophoblasts that attached to the explants (87.6±12.4% in 20% O_2 vs. 88.5±9.9% in 2% O_2). But most of these cells aggregated on the tissue surface, and invasion was markedly reduced (Fig. 2 *D*). In this case interactions between the fetal and maternal cells were morphologically similar to those ob-

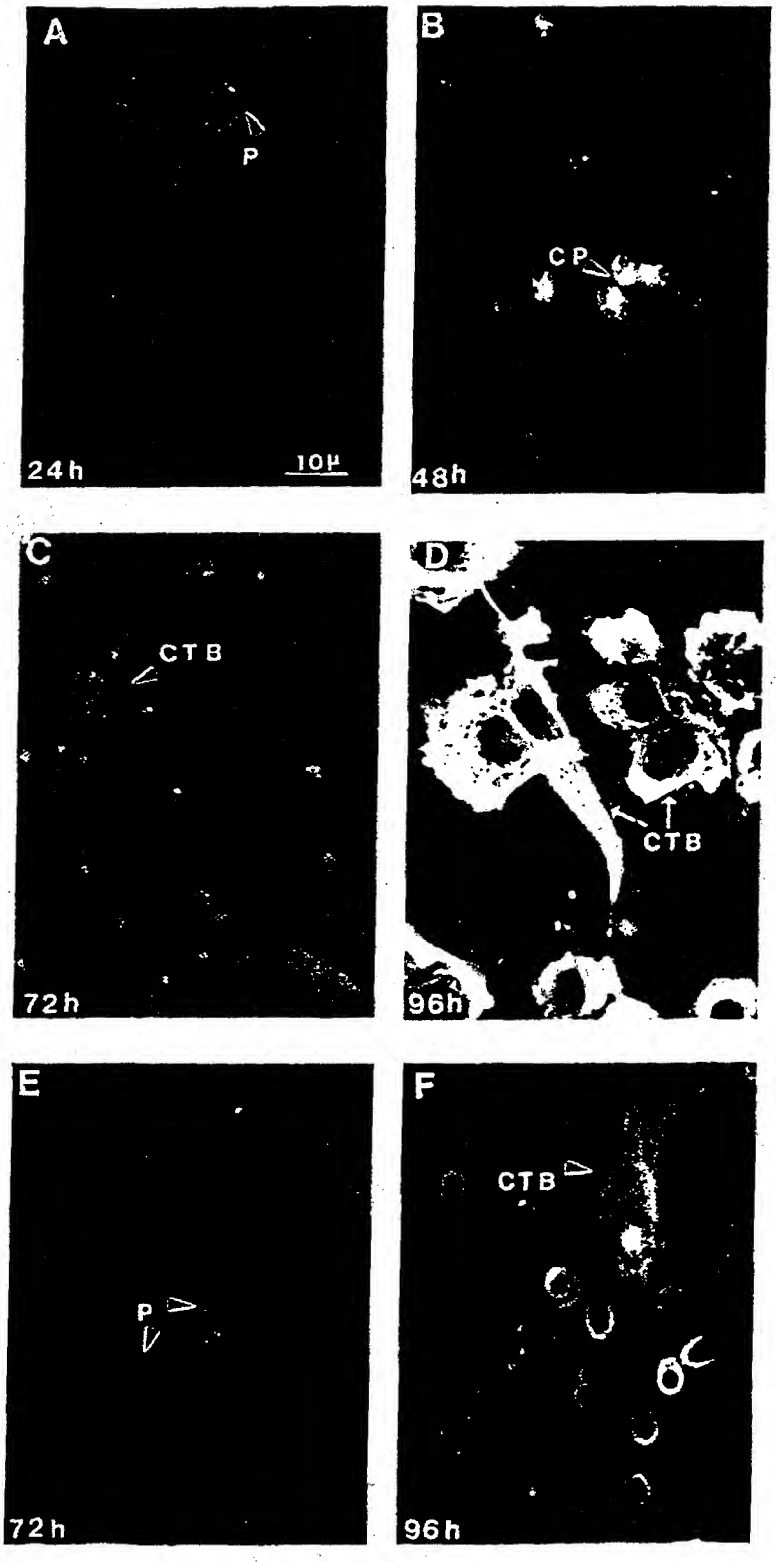

Figure 3. Reducing oxygen tension inhibited early gestation human cytotrophoblast invasion. Cytotrophoblasts isolated from pools of 10–12 wk placentas were cultured on Matrigel-coated (100±6 μm) Transwell filters. After 24, 48, 72, or 96 h the cultures were fixed and stained with anticytokeratin antibodies. The filters were removed from the inserts, inverted onto microscope slides and examined using a confocal microscope. We viewed 1-μm sections that were parallel to the membrane. The area we examined (50 μm total) included the underside as well as the upper surface of the filter and its Matrigel coating. Thus, the images appeared black unless fluorescently labeled (cytokeratin-positive) cells invaded to within 50 μm of the filter. (*A–D*) When the cultures were maintained in 20% oxygen, cytotrophoblasts (*CTB*) penetrated the Matrigel and emerged on the undersurface of the filter within 96 h of plating. By 24 h (*A*) fluorescent-labeled cells had started to invade the Matrigel. As a result, the emitted fluorescence was sufficient to make the filter surface appear light and the pores (*P*) appear black. Cytokeratin-positive cell processes (*CP*) were first seen in the pores at 48 h (*B*). By 72 h (*C*) many cells appeared near the original upper surface of the filter (*CTB*; *arrow heads*), and most of the filter pores contained cell processes. Between 72 (*C*) and 96 h (*D*) invasion proceeded rapidly, and entire cells (*CTB*; *arrows*) emerged on the filter underside. (*E* and *F*) When the cultures were maintained in 2% oxygen, invasion was significantly retarded. After 72 h (*E*) the filter surface still appeared black, indicating that cytokeratin-positive cells were not within the 50 μm area that was imaged. Cytotrophoblasts near the upper surface of the filter and cell processes in some of the pores were first visible at 96 h (*F*).

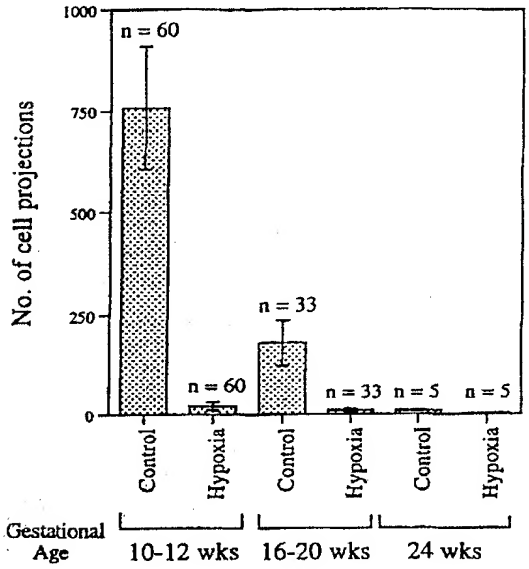

Figure 4. Quantifying the effects of hypoxia on early gestation human cytotrophoblast invasion. Cytotrophoblasts isolated from pools of 10–12, 16–20, or 24 wk placentas were cultured for 72 h on Matrigel-coated Transwell filters. Invasion was quantified by counting the number of cytokeratin-positive cell processes that penetrated the filter pores. As previously described, the invasiveness of control cells cultured under standard conditions (20% oxygen) decreased with advancing gestational age (6, 8). Culturing cytotrophoblasts isolated from either 10–12 or 16–20 wk placentas under hypoxic conditions (2% oxygen) significantly inhibited their invasiveness ($P < 0.001$ for 10–12 wk cells; $P < 0.001$ for 16–20 wk cells). *n*, number of filters. Three replicate filters were established from each isolation procedure, except that five filters from a single 24 wk placenta were analyzed. Error bars show SD.

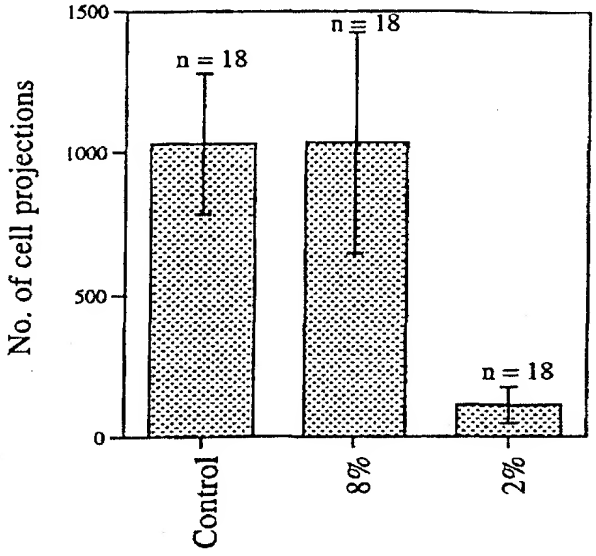

Figure 5. Cytotrophoblasts cultured in a 20 and an 8% oxygen atmosphere were similarly invasive. Cytotrophoblasts isolated from 10–12 wk placentas were cultured for 3 d on Matrigel-coated Transwell filters and invasion was quantified as described in Fig. 4. The cells were maintained under standard tissue culture conditions (20% O_2; *Control*), or in an atmosphere with an oxygen content comparable to that of the endometrium (8%), or under hypoxic conditions (2% O_2). The invasiveness of cells cultured in 20 and 8% O_2 was similar and significantly ($P < 0.01$) greater than that of cells maintained in a 2% O_2 atmosphere. *n*, number of filters. Six replicate filters were analyzed from three different isolation procedures. Error bars show SD.

served in tissue sections of the maternal-fetal interface in pregnancies complicated by preeclampsia (compare Fig. 2, *B* and *D*); the cells attached to the uterus, but invasion was shallow.

The other model we used allowed us to quantify differences in cytotrophoblast invasiveness and to determine whether hypoxia-induced changes required coculture with decidual explants. Purified 10–12 wk cytotrophoblasts were plated on the tops of Matrigel-coated Transwell filters (5, 6, 8, 9). After 24–96 h the filters were dissected from the inserts and mounted on slides such that their undersides faced upward. We used confocal microscopy to image the ECM, both sides of the filter and the pores that connected them. This showed that the thickness of the Matrigel averaged 100±6 μm. When cytotrophoblasts were plated in a 20% O_2 atmosphere, we observed the following progression. After the first 24 h of culture, cells had not penetrated the Matrigel to a sufficient depth to be visible in the area adjacent to the filter (Fig. 3 *A*). After 48 h, a few cells had invaded the Matrigel, and occasionally their processes traversed the pores (Fig. 3 *B*). However, by 72 h a large number of cells were in direct contact with the filter, and many pores contained processes (Fig. 3 *C*). After this stage invasion proceeded rapidly; at the end of the culture period (96 h) numerous cells had reached the underside of the filter (Fig. 3 *D*). In contrast, cytotrophoblasts that were cultured in 2% oxygen were much less invasive. By 72 h cells were only occasionally visible near the upper surface of the filter (Fig. 3 *E*). Even by 96 h, few processes had penetrated the pores or reached the filter underside (Fig. 3 *F*).

Next, we quantified the inhibitory effects of hypoxia on cytotrophoblast invasion by counting the number of cytokeratin-positive cell processes that extended through the filter after 72 h, the time when numerous cell processes had traversed the pores, but passive migration through previously formed tracks had not yet begun. We focused on gestational ages 8–24 wk because our previous studies showed that cytotrophoblast invasiveness peaks early in the first trimester and is greatly reduced by mid-gestation (6, 8). The results (Fig. 4) showed that there was a 90% reduction in invasion when cytotrophoblasts isolated from 10–12 wk placentas were plated in 2% as compared with 20% oxygen. Similar results were obtained when we assayed the invasive capacity of cells isolated from 16–20 wk placentas cultured in the two conditions. By 24 wk, little invasion was evident in either the control or experimental cultures. These results suggest that low oxygen tension inhibits invasion by its direct effects on early gestation cytotrophoblasts, rather than by an indirect mechanism mediated by the decidua.

We then assayed the effects on cytotrophoblast invasion of maintaining cells isolated from 10–12 wk placentas in an 8% oxygen atmosphere, comparable to that encountered by cells in the endometrium during the first trimester and in the intervillous space after 12 wk of gestation (39). As shown in Fig. 5, the number of cell projections that reached the underside of the filter was nearly identical to that observed when the cultures were maintained in a 20% oxygen atmosphere.

To determine whether the negative effects of hypoxia on

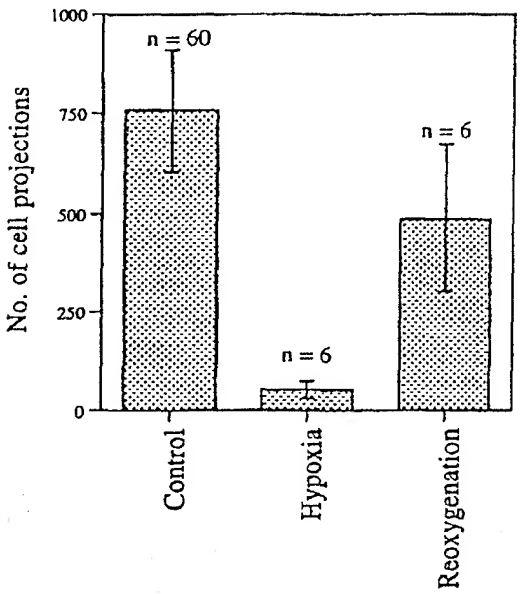

Figure 6. Reoxygenation reversed the negative effects of hypoxia on first trimester human cytotrophoblast invasion. Cytotrophoblasts isolated from 10–12 wk placentas were cultured for 6 d on Matrigel-coated Transwell filters and invasion was quantified as described in Fig. 4. The cells were either maintained in a 2% oxygen atmosphere for the entire period (*Hypoxia*) or transferred after 3 d to a standard tissue culture incubator (20% oxygen; *Reoxygenation*). In the latter case, invasion reached a level that was not significantly different from that of cells cultured for 3 d in a 20% oxygen environment (*Control*). *n*, number of filters. Three replicate filters were established from each isolation procedure. Error bars show SD.

cytotrophoblast (10–12 wk) invasion could be reversed, cells were either cultured for 6 d in 2% oxygen or switched after 3 d to a 20% oxygen atmosphere, where they were maintained for an additional 3 d. The results are shown in Fig. 6. As expected, cells that were continuously cultured under hypoxic conditions showed little ability to invade the ECM. In contrast, the invasiveness of cytotrophoblasts that were switched from 2 to 20% oxygen approached that of cells cultured under standard conditions for 3 d. The latter data (replicated from Fig. 4) were used for comparison purposes because nearly all of the early gestation cells that were cultured in a 20% oxygen atmosphere for 6 d had penetrated the pores and reaggregated on the bottoms of the filters. Thus, the short-term effects of hypoxia on cytotrophoblast invasion are reversible, suggesting that under some circumstances cytotrophoblasts may be relatively resistant to reoxygenation injury.

Hypoxia inhibits cytotrophoblast α1/β1 integrin expression. The third endpoint we assayed was the cells' expression of integrin cell–ECM receptors which are stage-specific markers for transitions in cytotrophoblast differentiation, as well as important regulators of the invasion process. Plating 10–12 wk gestation cytotrophoblast stem cells on Matrigel supports their differentiation along the invasive pathway, as assayed by the cells' ability to alter their integrin repertoire (9). Here, we used this culture system to determine whether hypoxia alters the normal pattern of cytotrophoblast integrin switching. As we showed previously (9), cytotrophoblast stem cells maintained in 20% O_2 rapidly upregulated their expression of integrins α5 (Fig. 7 *A*) and β1, as well as ECM constituents (collagen IV and A^+B^+ fibronectin) (data not shown). In vivo these molecules are first expressed just after the cells leave their basement membrane (3), i.e., during the initial stages of differentiation. Under standard culture conditions (20% oxygen), cytotrophoblasts also upregulated integrin α1 expression (Fig. 7 *C*). In vivo this occurs at a later point in the differentiation process, i.e., as the cells invade the uterus (3). The same results were obtained when the cells were cultured in an 8% oxygen atmosphere. Cytotrophoblasts cultured in 2% O_2 completed the initial stages of this differentiation program. They upregulated expression of α5 (Fig. 7 *B*) and β1 integrin subunits, as well as collagen IV and fibronectin production (data not shown). However, the majority of cytokeratin-positive cells failed to express α1β1 (Fig. 7 *E*), suggesting that they can initiate, but not complete, the normal integrin switching program.

Discussion

The results of these experiments, taken together, suggest that oxygen tension is one factor that regulates human cytotrophoblast differentiation along the invasive pathway. In a hypoxic environment, the cells carry out only the initial stages of their normal differentiation program. They upregulate DNA synthesis and express integrins and ECM molecules (e.g., α5/β1 and A^+B^+ fibronectin) in a pattern characteristic of cytotrophoblasts in the early stages of differentiation along the invasive pathway in vivo (3, 9). However, the latter stage of the differentiation process, in which cytotrophoblasts upregulate the expression of integrin α1/β1, is inhibited in a manner that is similar to the situation we have observed in preeclampsia (15, 16). Comparing the invasiveness of cytotrophoblasts cultured in 2 and 20% oxygen suggests that the changes in stage-specific antigen expression we observed had functional consequences: culturing cytotrophoblasts in a low oxygen tension environment greatly reduced their invasiveness.

These data indicate that hypoxia changes the balance between proliferation and differentiation in cytotrophoblasts. With regard to the cell cycle, our results are in agreement with decades-old morphological studies that describe marked proliferation of the cytotrophoblast layer in tissue sections of placentas from pregnancies complicated by preeclampsia (44), a finding that has recently been confirmed (12). Similarly, culturing chorionic villi under hypoxic conditions has been reported to result in cytotrophoblast proliferation (45), although this result has not been replicated (46). Whether oxygen tension affects passage through the cell cycle in other cells has not been widely studied. Limited evidence suggests the observed effects are cell-type specific. For example, hypoxia induces proliferation of vascular smooth muscle cells (47, 48), but leads to G_1 arrest in a variety of tumor cell lines (49). Likewise, hypoxia stimulates differentiation of erythrocytes (via induction of erythropoietin; 50) but inhibits that of 3T3-L1 fibroblasts (51).

Our findings also suggest that hypoxia changes the cells' expression of integrin α1, which is a stage-specific marker of cytotrophoblast differentiation. The effects of hypoxia on adhesion molecule expression in other systems are beginning to be studied. For example, cultured human umbilical vein endothelial cells subjected to low oxygen tension upregulate, after reoxygenation, endothelial-leukocyte adhesion molecule-1 and intercellular adhesion molecule-1 expression (26, 52). Conse-

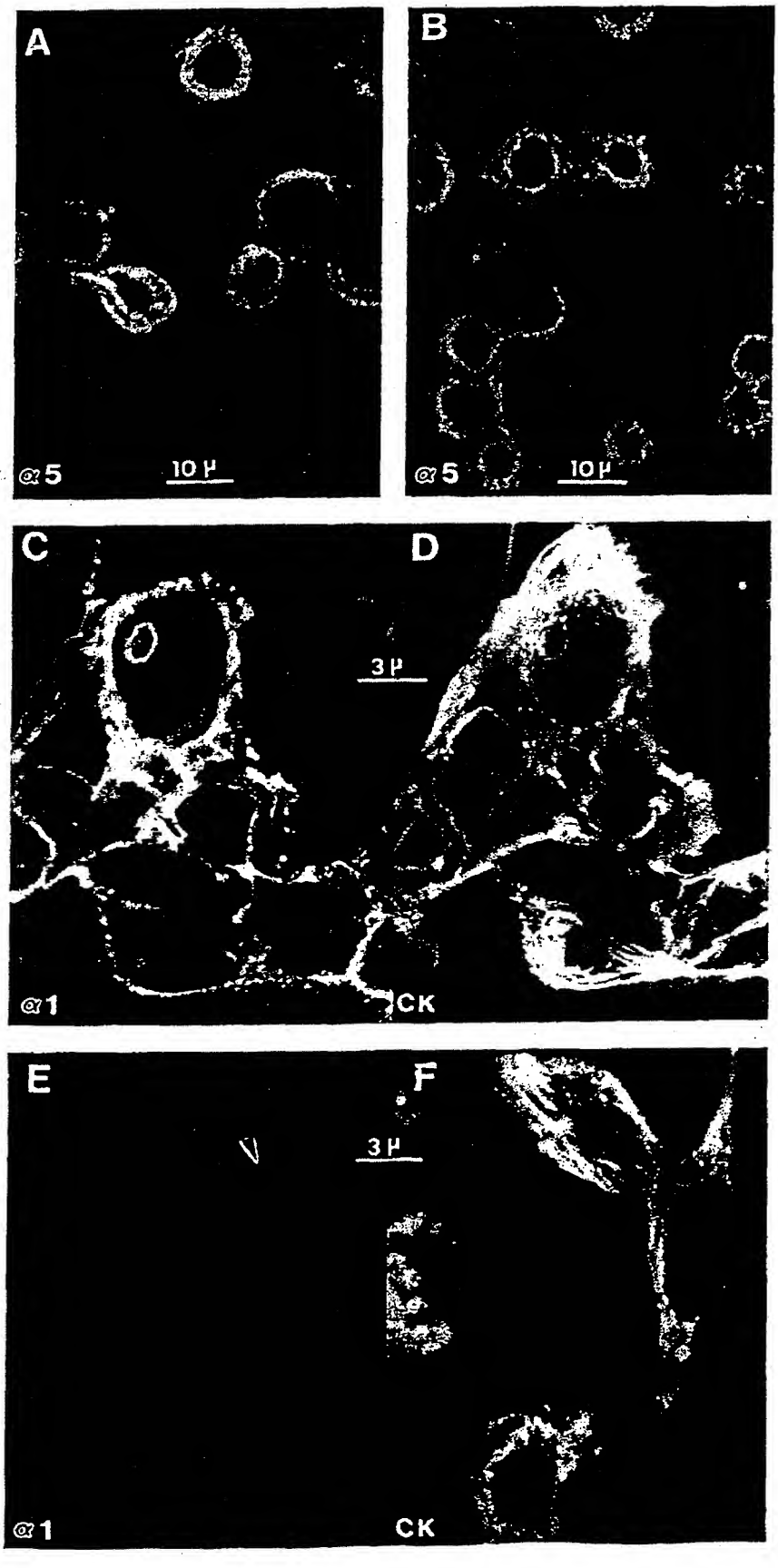

Figure 7. Hypoxia alters cytotrophoblast integrin expression. Isolated 10–12 wk cytotrophoblasts were plated on Matrigel-coated Transwell filters and cultured in 20 or 2% oxygen for 72 h. The cells were double stained with antibodies that recognized cytokeratin (*CK*) and either α5 or α1, two integrin subunits whose expression is upregulated during differentiation along the invasive pathway in vivo (3) and in vitro (9). Then the filters were removed from the inserts and mounted on slides such that the original Matrigel-coated surface faced upward. Integrin expression was analyzed by confocal microscopy. Greater than 98% of the cells cultured in either 2% (*A*) or 20% (*B*) oxygen reacted with the anti-α5 antibody. A similar percentage of cytotrophoblasts cultured in 20% oxygen upregulated integrin α1 expression (*C*). In contrast, the majority of cells that had been cultured in 2% oxygen did not react with the anti-α1 antibody (*E*). Often only weak reactivity was detected (*arrowhead*). The fact that the cells were cytotrophoblasts was verified by colocalizing integrin α1 and cytokeratin (*CK*) expression (*D* and *F*).

quently, there is a severalfold increase in the number of leukocytes that adhere, in a leukocyte function antigen (LFA)-1–dependent mechanism, to the reoxygenated human umbilical vein endothelial cell monolayers (52, 53). These results help explain why hypoxia leads to increases in leukocyte-blood vessel adhesive interactions. LFA-1 also mediates increased leukocyte adhesion to human muscle cells subjected to a low oxygen tension environment. In this case, intercellular adhesion due to vascular cell adhesion molecule-1 interactions with LFA-4 is simultaneously downregulated (53).

The concept that oxygen tension regulates cytotrophoblast differentiation has several interesting implications with regard to the interplay between physiology and placental development in normal pregnancy. The experiments described in this paper primarily focused on differentiation of cytotrophoblasts isolated from 10–12 wk human placentas. This time period is an important physiological transition in pregnancy during which blood flow to the placenta substantially increases. Morphological studies of the placental bed show that throughout much of the first trimester, cytotrophoblast invasion is confined to the terminal portions of uterine blood vessels near the uterine lumen (3). Ex vivo angiography of hysterectomy specimens (54) and in vivo color Doppler imaging (55) show no evidence of blood flow to the intervillous space during this same time period. During the second trimester of pregnancy, endovascular invasion proceeds rapidly, and these vessels are transformed into wide-bore, low-resistance, high-capacity channels.

Our data suggest that this physiological transition coincides with an important transition in placental development, namely the point at which cytotrophoblast differentiation along the invasive pathway becomes sensitive to hypoxia. Since we cannot obtain enough placental tissue to study the isolated cells before 8 wk gestation, we are using another invasion/differentiation model, explanting anchoring villi to Matrigel, for this purpose (42). These experiments show that before 7 wk gestation, hypoxia does not inhibit cytotrophoblast invasion (Genbacev, O., and S. J. Fisher, manuscript in preparation); such inhibition appears at ~ 10 wk gestation. These data are in accord with the results described here which show that hypoxia arrests cytotrophoblasts isolated from 10–12 wk placentas in the initial stages of differentiation along the invasive pathway. Thus, we hypothesize that during early pregnancy, allocation of the trophoblast lineage and differentiation/invasion can occur in a relatively hypoxic environment. However, normal placental development after the first trimester of pregnancy requires that the cytotrophoblast subpopulation that lies at the maternal–fetal interface has an adequate supply of maternal blood. Thus, our work adds new information as to why the uterine vascular changes that occur at the end of the first trimester and that substantially increase blood flow to the maternal–fetal interface are critical to the outcome of pregnancy.

With regard to abnormal placental development, we hypothesize that when cytotrophoblasts do not access an adequate maternal blood supply, they survive because they are relatively resistant to hypoxia. However, the cells are less able to invade, thus setting up the series of events that results in shallow placentation and, by some unknown mechanism, initiates the preeclampsia syndrome. The fact that we can duplicate the abnormalities in cytotrophoblast differentiation that occur in preeclampsia by culturing normal cells in a hypoxic environment suggests that the placental defects associated with this syndrome can be at least partially attributed to decreased placental perfusion and that this is an important proximal event in the chain of events that leads to this disease.

Acknowledgments

We thank Ms. Evangeline Leash for editing the manuscript.

This work was supported by National Institutes of Health grants HD 30367 and HD 22210 and by grant 3 RT-0324 from the University of California Tobacco-related Disease Research Program.

References

1. Fisher, S. J., and C. H. Damsky. 1993. Human cytotrophoblast invasion. *Semin. Cell Biol.* 4:183–188.

2. Cross, J. C., Z. Werb, and S. J. Fisher. 1994. Implantation and the placenta: key pieces of the development puzzle. *Science (Wash. DC).* 266:1508–1518.

3. Damsky, C. H., M. L. Fitzgerald, and S. J. Fisher. 1992. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway in vivo. *J. Clin. Invest.* 89:210–222.

4. Polette, M., B. Nawrocki, A. Pintiaux, C. Massenat, E. Maquoi, L. Volders, J. P. Schaaps, P. Birembaut, and J. M. Foidart. 1994. Expression of gelatinases A and B and their tissue inhibitors by cells of early and term human placenta and gestational endometrium. *Lab. Invest.* 71:838–846.

5. Fisher, S. J., T. Y. Cui, L. Zhang, L. Hartman, K. Grahl, G. Y. Zhang, J. Tarpey, and C. H. Damsky. 1989. Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *J. Cell Biol.* 109:891–902.

6. Librach, C. L., Z. Werb, M. L. Fitzgerald, K. Chiu, N. M. Corwin, R. A. Esteves, D. Grobelny, R. Galardy, C. H. Damsky, and S. J. Fisher. 1991. 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J. Cell Biol.* 113:437–449.

7. Librach, C. L., S. L. Feigenbaum, K. E. Bass, T. Cui, N. Verastas, Y. Sadovsky, J. P. Quigley, D. L. French, and S. J. Fisher. 1994. Interleukin-1β regulates human metalloproteinase activity and invasion in vitro. *J. Biol. Chem.* 269: 17125–17131.

8. Bass, K. E., D. Morrish, I. Roth, D. Bhardwaj, R. Taylor, Y. Zhou, and S. J. Fisher. 1994. Human cytotrophoblast invasion is up-regulated by epidermal growth factor: evidence that paracrine factors modify this process. *Dev. Biol.* 164:550–561.

9. Damsky, C. H., C. L. Librach, K. H. Lim, M. L. Fitzgerald, M. T. McMaster, M. Janatpour, Y. Zhou, S. K. Logan, and S. J. Fisher. 1994. Integrin switching regulates normal trophoblast invasion. *Development (Camb.).* 120:3657–3666.

10. McMaster, M. T., C. L. Librach, Y. Zhou, K.-H. Lim, M. J. Janatpour, R. DeMars, S. Kovats, C. H. Damsky, and S. J. Fisher. 1995. Human placental HLA-G expression is limited to differentiated cytotrophoblasts. *J. Immunol.* 154:3771–3778.

11. Roberts, J. M., R. N. Taylor, S. A. Friedman, and A. Goldfien. 1990. New developments in pre-eclampsia. *In* Fetal Medical Review. W. Dunlop, editor. Edward Arnold Publishers, London. 125–141.

12. Redline, R. W., and P. Patterson 1995. Pre-eclampsia is associated with an excess of proliferative, immature intermediate trophoblasts. *Human Pathol.* 26:594–600.

13. Lunell, N. O., L. E. Nylund, R. Lewander, and B. Sarby. 1982. Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera. *Clin. Exp. Hypertens.* 1:105–117.

14. Redman, C. W. 1991. Current topic: pre-eclampsia and the placenta. *Placenta.* 12:301–308.

15. Zhou, Y., C. H. Damsky, K. Chiu, J. M. Roberts, and S. J. Fisher. 1993. Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. *J. Clin. Invest.* 91:950–960.

16. Lim, K.-H., K. Bass, S.-H. Chun, C. H. Damsky, and S. J. Fisher. 1994. Differentiation of invasive cytotrophoblasts in normal and abnormal pregnancy. *Soc. Gynecol. Invest.* 42:107*a* (Abstr.)

17. Anderson, G. R., D. L. Stoler, and L. A. Scarcello. 1989. Normal fibroblasts responding to anoxia exhibit features of the malignant phenotype. *J. Biol. Chem.* 264:14885–14892.

18. Heacock, C. S., and R. M. Sutherland. 1986. Induction characteristics of oxygen regulated proteins. *Int. J. Radiat. Oncol. Biol. Phys.* 12:1287–1290.

19. Sciandra, J. J., J. R. Subjeck, and C. S. Hughes. 1984. Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation. *Proc. Natl. Acad. Sci. USA.* 81:4843–4847.

20. Vass, K., W. J. Welch, and T. J. Nowak. 1988. Localization of 70-kDa stress protein induction in gerbil brain after ischemia. *Acta Neuropathol.* 77: 128–135.

21. Gething, M. J., and J. Sambrook. 1992. Protein folding in the cell. *Nature (Lond.).* 355:33–45.

22. Beru, N., J. McDonald, C. Lacombe, and E. Goldwasser. 1986. Expres-

sion of the erythropoietin gene. *Mol. Cell. Biol.* 6:2571–2575.

23. Bondurant, M. C., and M. J. Koury. 1986. Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. *Mol. Cell. Biol.* 6:2731–2733.

24. Kourembanas, S., R. L. Hannan, and D. V. Faller. 1990. Oxygen tension regulates the expression of the platelet-derived growth factor-B chain gene in human endothelial cells. *J. Clin. Invest.* 86:670–674.

25. Kourembanas, S., P. A. Marsden, L. P. McQuillan, and D. V. Faller. 1991. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J. Clin. Invest.* 88:1054–1057.

26. Shreeniwas, R., S. Koga, M. Karakurum, D. Pinsky, E. Kaiser, J. Brett, B. A. Wolitzky, C. Norton, J. Plocinski, W. Benjamin, et al. 1992. Hypoxia-mediated induction of endothelial cell interleukin-1 alpha. An autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface. *J. Clin. Invest.* 90:2333–2339.

27. Karakurum, M., R. Shreeniwas, J. Chen, D. Pinsky, S. D. Yan, M. Anderson, K. Sunouchi, J. Major, T. Hamilton, K. Kuwabara, et al. 1994. Hypoxic induction of interleukin-8 gene expression in human endothelial cells. *J. Clin. Invest.* 93:1564–1570.

28. Longo, L. D., S. Packianathan, J. A. McQueary, R. B. Stagg, C. V. Byus, and C. D. Cain. 1993. Acute hypoxia increases ornithine decarboxylase activity and polyamine concentrations in fetal rat brain. *Proc. Natl. Acad. Sci. USA.* 90: 692–696.

29. Shweiki, D., A. Itin, D. Soffer, and E. Keshet. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature (Lond.).* 359:843–845.

30. Graven, K. K., R. F. Troxler, H. Kornfeld, M. V. Panchenko, and H. W. Farber. 1994. Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J. Biol. Chem.* 269:24446–24453.

31. Hassoun, P. M., F. S. Yu, A. L. Shedd, J. J. Zulueta, V. J. Thannickal, J. J. Lanzillo, and B. L. Fanburg. 1994. Regulation of endothelial cell xanthine dehydrogenase and xanthine oxidase gene expression by oxygen tension. *Am. J. Physiol.* L163–L171.

32. Murphy, B. J., K. R. Laderoute, R. J. Chin, and R. M. Sutherland. 1994. Metallothionein IIA is up-regulated by hypoxia in human A431 squamous carcinoma cells. *Cancer Res.* 54:5808–5810.

33. Semenza, G. L., and G. L. Wang. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* 12:5447–5454.

34. Wang, G. L., and G. L. Semenza. 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. USA.* 90:4304–4308.

35. Semenza, G. L., P. H. Roth, H. M. Fang, and G. L. Wang. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.* 269:23757–23763.

36. Webster, K. A., D. J. Discher, and N. H. Bishopric. 1993. Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxic cardiac myocytes. *J. Biol. Chem.* 268:16852–16858.

37. Koong, A. C., E. Y. Chen, and A. J. Giaccia. 1994. Hypoxia causes the activation of nuclear factor kappa B through the phosphorylation of I kappa B alpha on tyrosine residues. *Cancer Res.* 54:1425–1430.

38. Benjamin, I. J., B. Kroger, and R. S. Williams. 1990. Activation of the heat shock transcription factor by hypoxia in mammalian cells. *Proc. Natl. Acad. Sci. USA.* 87:6263–6267.

39. Rodesch, F., P. Simon, C. Donner, and E. Jauniaux. 1992. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet. Gynecol.* 80:283–285.

40. Charbonneau, H., N. K. Tonks, S. Kumar, C. D. Diltz, M. Harrylock, D. E. Cool, E. G. Krebs, E. H. Fischer, and K. A. Walsh. 1989. Human placenta protein-tyrosine-phosphatase: amino acid sequence and relationship to a family of receptor-like proteins. *Proc. Natl. Acad. Sci. USA.* 86:5252–5256.

41. Kovats, S., C. Librach, P. Fisch, E. K. Main, P. M. Sondel, S. J. Fisher, and R. DeMars. 1991. Expression and possible function of the HLA-G a chain in human cytotrophoblasts. *In* Cellular and Molecular Biology of the Materno-Fetal Relationship. Vol. 212. G. Chaouat, and J. Mowbray, editors. John Libbey Eurotext Ltd., New York. 21–29.

42. Genbacev, O., S. A. Schubach, and R. K. Miller. 1992. Villous culture of first trimester human placenta—model to study extravillous trophoblast (EVT) differentiation. *Placenta.* 13:439–461.

43. Genbacev, O., S. S. Powlin, and R. K. Miller. 1994. Regulation of human extravillus trophoblast (EVT) cell differentiation and proliferation *in vitro*. Role of epidermal growth factor (EGF). *Troph. Res.* 8:427–442.

44. Fox, H. 1964. The villous cytotrophoblast as an index ischaemia. *J. Obstet. Gynaecol. Br. Commonw.* 71:885–893.

45. Tominaga, T., and E. W. Page. 1966. Accommodation of the human placenta to hypoxia. *Am. J. Obstet. Gynecol.* 94:679–691.

46. Burton, G. J., T. M. Mayhew, and L. A. Robertson. 1989. Stereological re-examination of the effects of varying oxygen tensions on human placental villi maintained in organ culture for up to 12 h. *Placenta.* 10:263–273.

47. Butler, A. J., M. J. Eagleton, D. Wang, R. L. Howell, A. R. Strauch, V. Khasgiwala, and H. C. Smith. 1991. Induction of the proliferative phenotype in differentiated myogenic cells by hypoxia. *J. Biol. Chem.* 266:18250–18258.

48. Dempsey, E. C., I. F. McMurtry, and R. F. O'Brien. 1991. Protein kinase C activation allows pulmonary artery smooth muscle cells to proliferate to hypoxia. *Am. J. Physiol.* 260:L136–L145.

49. Graeber, T. G., J. F. Peterson, M. Tsai, K. Monica, A. J. Fornace, and A. J. Giaccia. 1994. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. *Mol. Cell. Biol.* 14:6264–6277.

50. Goldberg, M. A., C. C. Gaut, and H. F. Bunn. 1991. Erythropoietin mRNA levels are governed by both the rate of gene transcription and posttranscriptional events. *Blood.* 77:271–277.

51. Sahai, A., M. S. Patel, A. S. Zavosh, and R. L. Tannen. 1994. Chronic hypoxia impairs the differentiation of 3T3-L1 fibroblast in culture: role of sustained protein kinase C activation. *J. Cell. Physiol.* 160:107–112.

52. Arnould, T., C. Michiels, and J. Remacle. 1993. Increased PMN adherence on endothelial cells after hypoxia: involvement of PAF, CD18/CD11b, and ICAM-1. *Am. J. Physiol.* C1102–C1110.

53. Ginis, I., S. J. Mentzer, and D. V. Faller. 1993. Oxygen tension regulates neutrophil adhesion to human endothelial cells via an LFA-1-dependent mechanism. *J. Cell. Physiol.* 157:569–578.

54. Hustin, J., and J. P. Schaaps. 1987. Echographic [corrected] and anatomic studies of the maternotrophoblastic border during the first trimester of pregnancy [published erratum appears in *Am. J. Obstet. Gynecol.* 1987. 157: 1291]. *Am. J. Obstet. Gynecol.* 157:162–168.

55. Jaffe, R., and J. J. Woods. 1993. Color Doppler imaging and in vivo assessment of the anatomy and physiology of the early uteroplacental circulation. *Fertil. Steril.* 60:293–297.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/47, 14/775, C12N 5/08, G01N 33/68, 33/574, 33/92

A2

(11) International Publication Number: WO 96/33214

(43) International Publication Date: 24 October 1996 (24.10.96)

(21) International Application Number: PCT/US96/05441

(22) International Filing Date: 18 April 1996 (18.04.96)

(30) Priority Data:
08/423,409 18 April 1995 (18.04.95) US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, Oakland, CA 94612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FISHER, Susan, J. [US/US]; 1347 4th Avenue, San Francisco, CA 94122 (US). GENBACEV, Olga [YU/US]; 1050 Crestview Drive #45G, Mountain View, CA 94040 (US). FOULK, Russell [US/US]; 740 Alta Vista, Pacifica, CA 94044 (US). CLAUSER, Karl, R. [US/US]; 3200 Treetops Circle, San Bruno, CA 94066 (US). BURLINGAME, Alama, L. [US/US]; 26 Alexander Avenue, Sausalito, CA 94965 (US).

(74) Agents: HUNTER, Tom et al.; Townsend and Townsend and Crew, Steuart Street Tower, One Market, San Francisco, CA 94105-1492 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

(57) Abstract

This invention is the discovery that hypoxic mammalian fetal trophoblast cells are a valid model of abnormal maternal plancental function and of other invasive cell types. The trophoblast cells differentially express a number of proteins when grown under hypoxic conditions. These proteins are useful for determining the health of the trophoblasts, the condition of a maternal placental interface and the invasive properties of metastatic and other invasive cell types. The hypoxic cytotrophoblast cells provide a convenient means to isolate these proteins and develope assay kits based upon the presence or absence of the proteins in a human test sample.

ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application Serial No. 08/423,409 filed April 18, 1995 which is incorporated herein by reference for all purposes.

FIELD OF THE INVENTION

The present invention relates to the field of reproductive biology. More particularly, the present invention relates to particular proteins whose expression is differentially regulated in hypoxic trophoblasts.

BACKGROUND OF THE INVENTION

Implantation and placental development (placentation) involve a complex series of physiological events that result in the physical connection between the mammalian embryo and its mother. Failures in implantation and placental development are clinically important. About one-third of normal human pregnancies end in spontaneous abortion, with 22% of such abortions occurring before pregnancy is even detected clinically (Wilcox *et al. N. Engl. J. Med.*. 319: 189 (1988)). The actual abortion rate may be even higher as failures in development during the peri-implantation period account for almost 80% of embryonic loss that occurs in farm animal and, by implication, other species (Roberts, *et al. Oxf. Rev. Reprod. Biol.*, 12: 147 (1990), Roberts *et al.*, *Endocr. Rev.*, 13: 432 (1992), King, *Can. Vet. J.*, 32: 99 (1991). Even seemingly minor defects in placentation can have severe negative consequences. In humans, for example, abnormalities in the vascular connections can result in preeclampsia.

Preeclampsia is an idiopathic, life-threatening disease of late pregnancy in which hypertension is associated with hepatic, neurologic, hematologic, or renal involvement. Rapid development of edema, particularly of the ankles, face and hands,

along with a rise in blood pressure, usually signals the onset of this condition. Jaundice and abnormal liver function may be present.

The rapid acceleration of blood pressure elevation is accompanied by an increase in proteinuria, oliguria, edema, and coagulopathy. This is a life threatening syndrome and tends to recur with future pregnancies. At the same time, the baby's normal development slows with accompanying intrauterine growth retardation. In the postpartum period, proteinuric patients are particularly susceptible to the development of postpartum renal failure. Preeclampsia is often characterized by hyperreflexia, visual disturbances and headache indicating neurologic involvement which may ultimately progress to eclampsia characterized by convulsions. Preeclampsia occurs in 7-10% of pregnancies and is responsible for significant maternal and fetal morbidity (Roberts, *Pregnancy-related hypertension.*, pages 703-752 In *Maternal-Fetal Medicine-Principles and Practice*, Creasy & Resnick, eds., W.B. Saunders, Philadelphia (1984).

Once preeclampsia is diagnosed, hospitalization is indicated, since, as described above, the disease can rapidly progress to eclampsia, characterized by convulsions resulting in significant maternal and fetal trauma. The definitive treatment of preeclampsia and eclampsia is delivery of the conceptus, which is carried out promptly, if fetal size and maturity are adequate. If the fetus is immature, management consists of bed rest in a quiet environment and control of neurologic manifestations and blood pressure, the former with magnesium sulfate and the latter usually with vasodilators such as hydralazine and methyldopa. Early detection of preeclampsia and implementation of appropriate therapeutic measures greatly reduces maternal and fetal morbidity. The long term prognosis of promptly detected preeclampsia is generally favorable.

Despite decades of interest and research, the pathogenesis of this disease is still poorly understood. In recent years, however, the availability of the measurement of placental proteins opened new perspectives in the diagnosis of fetoplacental dysfunction. In particular, placental proteins have attracted interest as diagnostic markers of various pathologies during pregnancy. Thus, for example, a decrease in the maternal serum concentration of pregnancy-specific beta-l-glycoprotein (sp_1) detected by serial measurements could predict fetal malnutrition (Csaba, *Med. Sci.*, 10: 840-842 (1982); Karg *et al. Arch. Gynaekol*, 231: 67-73 (1981).

Biochemical tests of fetoplacental well-being have been applied in the study of preeclampsia (Chard *et al.*, pages 1-93 In: *Placental Function Tests*, Berlin, Springer-Verlag (1982)), sometimes with dramatic clinical significance (Lindberg, *et al. J. Obstet. Gynecol. Br. Commonw.*, 80: 1046-1053 (1973); Spellacy *et al. Am. J. Obstet. Gynecol.*, 109: 588-98 (1971)). However, despite the recent focus on molecular events underlying preeclampsia, relatively little is known regarding the etiology of this and related diseases of pregnancy.

SUMMARY OF THE INVENTION

The present invention provides an *in vitro* culture system that effectively models abnormal placental function characteristic of an abnormal maternal-placental interface. It was a discovery of the present invention that trophoblast cells cultured under hypoxic conditions attain a morphology, antigenic phenotype, and activity that appears identical to that observed in trophoblasts of an abnormal maternal-placental interface characteristic of various diseases of pregnancy such as threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. In particular, the cells show a decrease in expression levels of $\alpha 1/\beta 1$ integrins, the 92 kDa type IV collagenase and HLA-G whose upregulation is characteristic of normal trophoblasts, *in vivo,* and trophoblasts cultured identically under normal oxygen conditions.

In addition it was discovered that trophoblasts and explanted chorionic villi cultured under hypoxic conditions alter the expression levels of other, previously unidentified, proteins. These proteins have been isolated and characterized, in terms of molecular weight, pI and whether they are upregulated or down regulated under hypoxic conditions as compared to trophoblasts cultured identically under normal conditions (see Tables 1 and 2). In addition, a number of these proteins have been sequenced and identified in searches of a protein sequence database.

In view of these discoveries, this invention provides for proteins expressed by a mammalian, more preferably a primate or a human, fetal trophoblast cell, or chorionic villus, whose level of expression is substantially altered when the cell, or chorionic villus, is grown under hypoxic conditions. Preferred hypoxic conditions, when referring to *in vitro* culture conditions refer to culture under an atmosphere comprising

less than about 20% oxygen, more preferably less than about 10% oxygen and most preferably less than about 2% oxygen. Hypoxic conditions may include full anoxia (0% oxygen). Preferred hypoxic conditions also include conditions in which the expression levels of $\alpha 1/\beta 1$, the 92 kDa type IV collagenase and HLA-G whose, as well as cell invasiveness, are significantly decreased as compared to trophoblasts cultured identically under normal conditions. Particularly preferred proteins include one or more of the proteins described in Table 1 and Table 2 as having increased or decreased release under hypoxic conditions.

This invention additionally provides for methods of culturing trophoblasts and/or chorionic villi under hypoxic conditions as described above, and below in the specification. The method may further comprise measuring the expression of a protein selected from the proteins listed in Table 1 and Table 2 as having increased or decreased release under hypoxic conditions.

The proteins expressed by the hypoxic trophoblasts or the hypoxic chorionic villi of the present invention can be used as markers indicative of the presence of hypoxic conditions which in turn, are indicative of an abnormal maternal-placental interface and consequent abnormal placental function. Thus this invention provides for a method of detecting hypoxic trophoblast cells, or hypoxic chorionic villi, comprising measuring the expression level of a protein whose expression is altered in hypoxic trophoblasts as compared to normal (normoxic) trophoblasts or whose expression is altered in hypoxic chorionic villi as compared to normal (normoxic) chorionic villi. In a particularly preferred embodiment, the protein is one or more proteins selected from the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. Most preferred proteins include human apolipoprotein A-1 (apo A-1), placental lactogen, chorionic gonadotropin, and fibrinogen. Preferred detection methods include direct detection of the protein, or alternatively, detection of mRNA encoding the protein. Particularly preferred protein detection methods include immunoassays, while preferred mRNA detection methods include quantitative amplification techniques (*e.g.* quantitative PCR), or hybridization methods such as Northern blots.

The present invention also provides a similar assay for detecting abnormal placental function. This involves analyzing a biological sample from a pregnant mammal, more preferably a pregnant primate or human, for abnormal expression of one or more proteins whose expression is altered in a hypoxic trophoblast. Particularly preferred

proteins are one or more proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. As indicated above, the hypoxic trophoblast, abnormal placental function, or abnormal maternal-placental interface are indicative or symptomatic of one or more diseases of pregnancy. Such diseases include, but are not limited to threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia.

The present invention also provides methods of screening for agents that mitigate the effects of an abnormal maternal-placental interface. These methods involve culturing trophoblasts or chorionic villi under hypoxic conditions (as described above) in the presence of the agent and assaying for changes in the phenotype of the hypoxic trophoblasts, or hypoxic villi, relative to hypoxic trophoblasts or hypoxic chorionic villi cultured without the presence of the agent. The step of assaying for changes in the phenotype may comprise measuring the invasiveness of the trophoblast. Alternatively, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed (released) by the trophoblasts. Proteins whose expression (release) is typically altered in hypoxic trophoblasts are preferably assayed, while the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions, are most preferably assayed.

Conversely, this invention also provides for methods of screening for agents that might induce the formation of an abnormal maternal-placental interface, or abnormal placental function. In this case, the trophoblasts, or chorionic villi, are cultured under normal conditions and assayed for changes in phenotype as compared to normal and/or hypoxic trophoblasts. Phenotypic changes similar to hypoxic trophoblasts or hypoxic chorionic villi are indicative of possible interference or alteration of the maternal-placental interface by the agent. As described above, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed by the trophoblasts or chorionic villi. Proteins whose expression is typically altered in hypoxic trophoblasts are preferably assayed, while the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions are most preferably assayed.

This invention additionally provides a method of modeling, *in vitro*, an abnormal maternal-placental interface or abnormal placental function. The method comprises culturing trophoblast cells in a hypoxic environment as described above.

Finally, this invention provides methods for identifying proteins that are indicative of metastasis and methods for detecting metastatic cells. Methods for identifying proteins indicative of metastasis involve culturing trophoblasts or chorionic villi under hypoxic conditions, as described above; detecting proteins that demonstrate an altered expression level as a result of the hypoxic conditions; and determining if these or related proteins are present in metastatic cells. Preferred proteins are one or more of the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. Preferred methods of determining if the proteins are present involve using antibodies specific for a protein whose expression is altered in hypoxic trophoblasts, more preferably an antibody specific for one of the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions.

Methods for detecting metastatic cells involve analyzing a biological sample from a mammal, more preferably a primate or human, for abnormal expression of one or more proteins indicative of metastasis. These preferably include proteins whose expression is altered in a hypoxic trophoblast or hypoxic chorionic villus, or closely related proteins. Particularly preferred proteins are one or more proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions.

Definitions

The term "invasiveness", as used herein refers to the ability of a cell to penetrate an extracellular matrix. Methods of measuring invasiveness are well known to those of skill in the art (see, *e.g.*, Librach *et al. J. Cell. Biol.*, 113: 437-449 (1991)) Similarly, an invasive cell type or an "invasive cell" refer to a cell capable of penetrating a tissue other than the tissue from which the cell originates. Invasive cells include, but are not limited to trophoblast and malignant cancer cells.

The term "protein", when used herein refers to a chain of amino acids whose α carbons are linked through peptide bonds. Proteins include native proteins *in vivo* or isolated native proteins. Proteins also include chemically or recombinantly synthesized proteins. In addition, it is to be understood that the term proteins, as used herein includes the protein product as translated from an mRNA molecule as well as the

protein products as subsequently modified. Thus proteins also include modified proteins such as glycoproteins, lipopoproteins and the like.

The term "normal oxygen conditions" refers to conditions under which a cells are exposed to the oxygen concentration that they experience *in vivo* in a normal healthy organism. Similarly, the term "normoxic cells" refers to cells exposed to normal oxygen conditions. When trophoblasts are cultured *in vivo* under normal atmospheric oxygen concentrations (about 20%, which, at a standard atmospheric pressure of 760 mm Hg, corresponds to a partial pressure of oxygen (pO_2) of about 152 mm Hg) the culture medium has an oxygen concentration of about 13% ($pO_2 \approx 98$ mm Hg) which corresponds approximately to the oxygen concentration of arterial blood. Thus, the term "normoxic" may be taken herein as refering to trophoblasts subject to an oxygen concentration comparable to trophoblasts cultured under an atmosphere containing a normal oxygen concentration (about 20% or about 152 mm Hg).

As used herein, an "immunoassay" is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is characterized by the use of specific binding to a particular antibody as opposed to other physical or chemical properties to isolate, target, and quantify the analyte.

The terms "expression" or "release" of a protein when used herein in reference to a protein whose expression or release is altered under hypoxic conditions are used to indicate that alterations in detectable protein level are due to alterations in the physiological activity of the cell or tissue and not to attribute a particular mechanism to the observed alteration in detectable protein level. Thus the phrases in "increase in expression" or "increase in release" of a protein are used to indicate that some action of the subject cell or tissue results in an increase in the detected levels of that protein, either released to the environment (*e.g.*, culture medium) or detected in a lysate. The increase can be due, for example, to increased expression of a gene encoding that protein, to defective expression of a native protein resulting in the detected protein "fragment", changes in uptake of the protein, changes in active secretion of the protein or changes in net release of the protein.

The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay

conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the trophoblast-secreted (trophoblast-released) proteins (TSPs) of the present invention and not to any other proteins present in a blood sample.

The phrase "a protein of spot n" as used herein, when referring to two dimensional electrophoresis gels describes a protein that occupies the same spatial position, in reference to other protein spots in an electrophoresis gel of the same type of cell or tissue preparation run under the same conditions as the referenced electrophoresis. The letter "n" refers to the spot number. Thus, for example, a protein of spot 7 will appear in a two-dimensional electrophoresis gel in the same position, in relation to the other spots, as the spot identified as spot 7 in Figures 1 and 2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 and Figure 2 show two-dimensional (PAGE) maps of unlabeled (Coomassie blue-stained) proteins released by chroionic villi grown in F12 HAM/DMEM (1:1/v:v) medium under standard (20% O_2, Figure 1) and hypoxic (2% O_2, Figure 2) conditions. Since the gels are 10% polyacrylamide, porteins in the lower molecular weight region of the gel are resolved best. As with cytotrophoblasts, the protein repertoire the cells release under both conditions is remarkably similar. Nevertheless, two types of changes were observed; abundance and pI. A small number of proteins were present in either greater or lesser amounts when the villi were maintained in 2% O_2. Protein abundance changes are characterized by the following symbols: ○ down in hypoxia; □ up in hypoxia; and Δ unchanged. Changes in apparent pI are attributed to changes in posttranslational modifications such as glycosylation and/or phosphorylation.

DETAILED DESCRIPTION

I. Hypoxic Trophoblasts or Hypoxic Chorionic Villi as Models of Abnormal Maternal-Placental Interface.

This invention involves the discovery that trophoblasts and/or chorionic villi grown under hypoxic conditions provide a useful *in vitro* model of the abnormal

maternal-placental interface and various disease states that are characteristic of, or result from, abnormal placental-maternal interaction or function. In particular, it is a discovery of the present invention that hypoxic trophoblasts express a gross morphology, a histology, an antigenic phenotype and a loss of invasiveness that is identical to that found in cells characteristic of the abnormal maternal-placental interface characteristic of a number of diseases of pregnancy. Such diseases include, but are not limited to, threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. The *in vitro* models of the present invention thus provides an excellent system in which to screen for therapeutic agents useful in the treatment of various diseases of pregnancy involving an abnormal maternal-placental interface.

In addition, the present invention provides for a number of proteins whose expression level is dramatically altered (either upregulated or downregulated) in hypoxic trophoblasts and hypoxic chorionic villi. These proteins provide useful markers for the early diagnosis of abnormal placental function and thus of diseases, such as preeclampsia, characteristic of abnormal placental function. As it is believed that these proteins are responsible for various complications of pregnancy including (1) an alteration in vascular reactivity associated with a hypersensitivity to infused angiotensin; (2) a decrease in production of prostacyclin with an associated increase in production of thromboxanes; (3) a decrease in renal hemodynamics, due at least in part to glomerular endotheliosis; (4) a widespread endothelial disorder, resulting in the loss of albumin from the intravascular space; and (5) an inherent immunologic misadaptation during placentation, resulting in incomplete trophoblast invasion of the spiral arterioles pathophysiological abnormalities. Detection of expression levels of these proteins provides a screening system for possible therapeutic agents that might mitigate these and other adverse effects of abnormal placental differentiation.

Finally, because of the similarity between trophoblasts and other invasive cell types (*e.g.* metastatic cells), the *in vitro* model of the present invention provides an excellent system for the identification of proteins that mediate cellular invasion of a tissue and for the screening of therapeutic agents that may inhibit the invasive activity of such cells.

A) The abnormal maternal-placental interface

The structure and function of the maternal-placental interface is mediated by the action of trophoblast cells. Trophoblasts are specialized epithelial cells of the placenta that physically connect the embryo and the uterus. In primates and rodents, trophoblasts are invasive, breaching uterine blood vessels and thereby achieving direct contact with maternal blood. In particular, human trophoblasts are extremely invasive; they traverse the uterine epithelium and invade the decidua, the inner third of the myometrium and the maternal arteries.

As used herein, the term "trophoblasts" includes the cytotrophoblast stem cells and lineages derived from these stem cells. The various lineages derived from cytototrophoblast stem cells are generally known to those of skill in the art. In humans, for example, two differentiation pathways exist for cytotrophoblasts, giving rise to populations that are morphologically and functionally distinct (Cross *et al. Science*, 266: 1508-1518 (1994). In the first trimester, cytotrophoblast stem cells reside in chorionic villi of two types; "floating" villi that do not contact the uterine wall and "anchoring" villi that do contact the uterine wall. Cytotrophoblasts in the floating villi exist only as polarized epithelial monolayers, anchored to a basement membranae and surrounding a stromal core containing fetal blood vessels. These cytotrophoblasts, which are highly proliferative in the first trimester of gestation, differentiate exclusively by fusing to form a syncytial layer that covers the villus. Floating villi, which make up the fetal compartment of the placenta, are bathed by maternal blood and perform gas and nutrient exchange functions.

In contrast, anchoring villi contain cytotrophoblast stem cells that enter both differentiation pathways. In much of the anchoring villus, cytotrophoblasts fuse to form a syncytium. However, at selected sites, cytotrophoblasts break through the syncytium and form multilayered columns of nonpolarized cells. Anchoring villi physically connect the embryo to the uterine wall via these cell columns, and give rise to the most highly invasive and migratory cytotrophoblasts. These invasive trophoblasts (also known as intermediate trophoblasts, cytotrophoblasts, or x-cells) invade uterine blood vessels.

B) *In vitro* model of the abnormal maternal-placental interface.

A large body of evidence indicates that preeclampsia, and other diseases of pregnancy, are associated with highly characteristic abnormalities in placental development (referred to herein has an "abnormal maternal-placental interface") such that the placenta is only superficially connected to the uterus. Cytotrophoblast invasion is shallow and does not proceed beyond the decidual portions of the spiral arteries. (Redman, *New Engl. J. Med.* 323: 478 (1990); Brosens *et al. Obster, Gynecol. Annu.* 1: 177 (1972) Gerretsen *et al., Brit. J. Obstet. Gynecol.*, 88: 876 (1981); Moodley and Ramsaroop, *S. Afr. Med. J.*, 75: 376 (1989)). In addition, not as many vessels show evidence of trophoblast invasion (Khong *et al. Br. J. Obstet. Gynecol.*, 93: 1049 (1986)).

These morphological differences are a dramatic contrast to normal development (placental differentiation) in which, as explained above, the trophoblasts, detach from their basement membranae, aggregate, and invade much of the uterus and its arterial system thereby forming an intimate connection (the maternal-placental interface) between the mother and the fetus. As used herein, the term "abnormal placental function" refers to the physiological consequences of this abnormal placental development.

It was a discovery of the present invention that hypoxia plays a role in this abnormal development process leading to superficial connection between the placenta and the uterus. Without being bound to a particular theory, it is believed that the lack of uterine blood vessel invasion by trophoblasts, which typically initiates maternal blood flow to the placenta in normal pregnancy, makes this tissue relatively hypoxic under preeclamptic conditions. The resulting hypoxia is associated with the differential regulation of a number of proteins that are released to the maternal circulation.

It was a discovery of the present invention that cytotrophoblasts cultured under hypoxic conditions (as described in Examples 1 and 2) showed abnormal differentiation that was *identical* to that observed in preeclampsia *in vivo*. The cells expressed the *same* altered pattern of stage-specific antigens as that seen *in vivo*. In particular, the hypoxic cells expressed very low levels of $\alpha 1/\beta 1$, the 92 kDa type IV collagenase and HLA-G whose upregulation is characteristic of normal cytotrophoblasts. Also as in preeclampsia, the cells' invasiveness was greatly reduced. This is the exact same antigenic phenotype that characterizes cytotrophoblast differentiation in preeclampsia and indicates that these hypoxic trophoblast cultures can be used to identify

unique proteins characteristic of an abnormal maternal-placental interface. Such proteins are candidates for the toxic placental factors that are thought to produce the maternal syndrome associated with preeclampsia and other diseases of pregnancy.

Similarly, it was also a discovery of this invention that chorionic villi (*e.g.*, anchoring chorionic villi) cultured under hypoxic conditions also showed altered release of proteins that can be used as markers of an abnormal maternal-placental interface. In particular, these proteins appear to be good markers of preeclampsia and other diseases of pregnancy.

Thus, in one embodiment, this invention provides for an *in vitro* model of abnormal maternal-placental interface or abnormal placental function. This model comprises culturing cytotrophoblasts and/or chorionic villi under hypoxic conditions. As used herein, hypoxic conditions are culture conditions under which the trophoblasts or chorionic villi are exposed to oxygen concentrations less than those to which they are exposed *in vivo* in a normal healthy pregnancy. When trophoblasts or chorionic villi are cultured under normal atmospheric oxygen concentrations (about 20% O_2) the culture medium contains about 13% oxygen ($pO_2 \approx 98$ mm Hg) which is comparable the physiological oxygen concentrations trophoblasts experience *in vivo* in a normal, healthy pregnancy. Thus, hypoxic conditions refers to culture conditions in which cells are grown under an atmosphere containing less than about 20% oxyten, more preferably less than about 10% oxygen and most preferably less than about 2% oxygen. As trophoblasts are capable of growing when fully anoxic, as used herein, hypoxic conditions include fully anoxic conditions.

The trophoblasts may be isolated and cultured according to any of a number of means well known to those of skill in the art. In a preferred embodiment, the trophoblast cells are isolated and cultured according to the methods described by Fisher *et al. J. Cell. Biol.*, 109: 891-902 (1989) and Librach *et al., J. Cell. Biol.*, 113: 437-449 (1991) and detailed in Example 1. Generally, first and second trimester placentas are obtained immediately after vacuum aspiration, and the chorionic villi are prepared as described by Fisher *et al. J. Cell. Biochem.*, 27: 31-41 (1985). The isolation of cytotrophoblast cells from villi is performed as described in Fisher *et al., Troph. Res.*, 4: 115-138 (1990). Briefly, the washed villus pellet is incubated for either 20 (first trimester) or 30 min (second trimester) (5:1 vol/wt/wt) in enzymatic dissociation solution I (PBS containing 500 U/ml collagenase [type IV: Sigma Chemical Col, St. Louis,

Missouri, USA], 200 U/ml hyaluronidase [type 1-S; Sigma Chemical Co.], 0.2 mg/ml Dnase [type IV; Sigma Chemical Co.], and 1 mg/ml BSA). The villi are separated from the supernatant, which contains the syncytium, by centrifugation and incubated for 10 minutes in dissociation solution II (PBS containing 0.25% trypsin [type XII; Sigma Chemical Co.], 2mM EDTA, and 0.2 mg/ml DNase). The dissociated cells are isolated by centrifugation, resuspended in 4 ml of medium containing 10% FCS and layered over a preformed Percoll gradient made up in Hanks' balanced salt solution according to the method of Kliman *et al.*, *Endocrinol.*, 118: 1567-1582 (1986). The gradient is centrifuged (1,000 x g) for 25 min at room temperature, after which a broad band in the middle of the tube containing the cytotrophoblast cells is removed. The cells are washed several times and resuspended in MEM D-valine medium (Gilbert and Migeon, *Cell*, 5: 11-17 (1975)) containing either 20% dialyzed FCS or 2% Nutridoma, 1% glutamine, and 50 μg/ml gentamicin.

Then 1 ml, containing about 5 x 10^5 cytotrophoblasts, is added per 15-mm tissue culture well. In most cases, either the wells or coverslips (12 mm diameter) placed within the wells, are coated with an extracellular matrix (ECM) produced by PF HR9 cells as described by Fisher *et al. J. Cell. Biochem.*, 25: 31-41 (1985). Under these conditions, the cells adhere to produce a confluent monolayer within 4 hours.

To isolate term cytotrophoblasts, the washed villus pellet is subjected to three cycles of trypsinization (dissociation solution II) and the resulting cells are purified on a Percoll gradient as described above.

Similarly, chorionic villi can be isolated and cultured according to standard methods known to those of skill in the art. For example, human chorionic villi can be disected from placentas (*e.g.*, first trimester placentas) and cultured in F12 HAM/DMEM (1:1/v:v) culture medium. Detailed culture methods are provided in Example 4.

One of skill will appreciate that other compositions may be added to the culture medium. These may include, but are not limited to, various labeled amino acids (*e.g.* radio-labeled amino acids such as [^{35}S]cysteine and [^{35}S]methionine, [^{3}H]leucine and the like) to facilitate the quantitative analysis of newly synthesized proteins, or various pharmacological agents to be tested for their effect on cytotrophoblast differentiation.

One of skill will appreciate that there are numerous variations of this isolation and culture procedure that may be utilized. However, where it is desired to

isolate proteins expressed by the cultured trophoblasts, it is preferred to use culture medium lacking serum.

II. Proteins Characteristic of Abnormal Placental Function.

It was also a discovery of the present invention that hypoxic trophoblasts and hypoxic chorionic villi express various proteins at altered levels as compared to the identical cells cultured under normoxic conditions. These proteins appear to be characteristic of hypoxic trophoblasts and/or hypoxic chorionic villi. Thus, in one embodiment, this invention provides for proteins expressed by mammalian fetal trophoblast cells and/or chorionic villi grown under hypoxic conditions as described above. In particular, this invention provides for proteins that are over or under-expressed as much as five-fold as compared to identical cells under normoxic conditions.

Proteins from hypoxic trophoblasts were isolated using 2-dimensional electrophoresis (2D SDS-PAGE) as according to the method of Patton *et al.* *Biotechniques* 8: 518(1990), with precautions suggested by Hunkapillar *et al. Methods in Enzymology,* 91: 227 (1983)as described in Example 2. After calculation of molecular weight from the PAGE gel, the isoelectric point (pI) was determined by isoelectric focussing. Hypoxic trophoblast proteins are listed in Table 1, which includes molecular weights, isoelectric points and whether the protein is upregulated or down regulated in hypoxic trophoblasts. Proteins identified from hypoxic chorionic villi are identified in Table 2 (*See,* example 3).

Table 1. Molecular weight and pI of proteins whose expression is altered in hypoxic trophoblasts.

Protein	Molecular Weight* (kDa)	pI	Expression
A	21	6.0	increased
B	22	7.0	increased
C	23	7.5	increased
D	55	8.5	increased
E	62	5.5	increased
F	40	4.5	decreased
G	67	6.5	decreased
H	75	9.0	decreased

* Molecular weights are estimated from electrophoretic gels (± 5 kDa).

The 2D gels gave no evidence of overlapping spots indicating that each of these proteins was fully isolated. One of skill in the art, will appreciate that the proteins may be further characterized by a number of means including, but not limited to, amino acid analysis and sequencing. Methods of further purification, amino acid analysis and sequencing are routine and well known in the art. These include, but are not limited to protein purification methods as described in Methods in Enzymology, *Guide to Protein Purification,* M. Deutscher, ed. Vol. 182 (1990), as well as various cloning and sequencing strategies as described by Sambrook, *et al., Molecular Cloning: A Laboratory Manual 2nd Ed.*, Vols. 1-3, Cold Spring Harbor Laboratory (1989); *Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques,* Berger and Kimmel, eds., San Diego: Academic Press,Inc. (1987)), or *Current Protocols in Molecular Biology*, Ausubel, *et al.*, eds., Greene Publishing and Wiley-Interscience, New York (1987).

In a particularly preferred embodiment, the proteins are initially isolated using a preparative 2D gel. The isolated proteins are then sequenced using mass spectrometry methods as detailed in Example 3.

Of course, the proteins listed in Table 1, are not the only proteins whose expression is altered in hypoxic cytotrophoblasts or hypoxic chorionic villi. The present invention thus provides for a method of identifying proteins whose expression is altered under hypoxic conditions. This method includes the steps of culturing cells, under

hypoxic conditions and detecting proteins whose expression is altered. In a preferred embodiment, the cells are trophoblast cells or cells in explanted chorionic villi, cultured as described above. The proteins whose expression is altered are preferably identified by 2D SDS-PAGE as described above and in Example 2.

III. Screening for an Abnormal Placental-Maternal Interface.

The proteins whose expression is altered in hypoxic trophoblasts (referred to herein as trophoblast-secreted or trophoblast-released proteins or TSPs) or in hypoxic chorionic villi (referred to as chorionic villus-released proteins), more preferably the proteins listed in Table 1, may be used as markers of abnormal placental function (*e.g.* as caused by an abnormal maternal-placental interface) and therefore as markers for the resulting diseases of pregnancy, such as preeclampsia. This invention thus provides for methods of detecting an abnormal placental-maternal interface, hypoxic trophoblasts, hypoxic chorionic villi, abnormal placental function, or a disease of pregnancy (*e.g.* preeclampsia) by detecting or measuring the expression levels of a protein whose expression level is altered in hypoxic trophoblasts or chorionic villi as compared to normoxic trophoblasts or normal chorionic villi, respectively. In a preferred embodiment, the method involves determining the expression level of one or more of the proteins listed in Table 1.

While the expression levels of the protein may be determined *in vivo,* in a preferred embodiment, they are determined *ex vivo* in a biological sample or in a culture derived from a biological sample. The proteins are preferably quantified in a biological sample derived from a patient. As used herein, a biological sample is a sample of biological tissue or fluid that contains a concentration of the protein being screened that may be correlated with *in vivo* trophoblast expression levels of the same protein. Particularly preferred biological samples include blood, chorion villus biopsy, amniocentesis and cervicovaginal secretions. See, for example, U.S. Patent No. 5,096,830, incorporated herein by reference, which describes cervicovaginal secretions as diagnostic assay samples, and provides means for taking such samples.

In another preferred embodiment, trophoblast-secreted proteins (TSPs) are quantified in whole blood or blood derivatives such as blood plasma or blood serum. Blood samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by venipuncture. Although the sample is typically

taken from a human patient, the assays can be used to detect trophoblast-secreted proteins in samples from any mammal, such as primates, rodents, canines, felines, bovines and porcines.

The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

In a preferred embodiment, assays are performed using blood plasma (blood lacking a cellular component) or blood serum (blood lacking a cellular component and clotting factors). Means of preparing blood plasma are well known to those of skill in the art and typically involve centrifugation or filtration to produce blood plasma, or clotting followed by centrifugation or filtration to produce blood serum. The blood plasma or serum may be diluted by the addition of buffers or other reagents well known to those of skill in the art and may be stored for up to 24 hours at 2-8°C, or at -20°C or lower for longer periods, prior to measurement of TSPs.

A) Quantification of trophoblast-secreted proteins.

The trophoblast-secreted proteins (TSPs) and proteins released by the chorionic villi of this invention may be detected and quantified by any of a number of means well known to those of skill in the art. These may include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, 2D electrophoresis as described in Example 2, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay(RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

As used herein, an immunoassay is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is characterized by the use of specific binding of the trophoblast secreted protein to a particular antibody as opposed to other physical or chemical properties to isolate, target, and quantify the analyte.

1) Antibodies to trophoblast- or chorionic villus-released proteins.

Antibodies can be raised to the trophoblast- or chorionic villus-released proteins of the present invention using routine methods well known to those of skill in the art. As used herein "antibodies" include immunoglobulin or a population of immunoglobins which specifically bind to an antigen. Thus an antibody may be monoclonal or polyclonal including individual, allelic, strain, or species variants, both in their naturally occurring (full-length) forms, various antibody fragmentary forms and in recombinant forms. Additionally antibodies is intended to refer to recombinantly expressed antibodies as in phage-display libraries. Antibodies can be raised to the trophoblast- or chorionic villus-released proteins in either their native configurations or in non-native configurations. Anti-idiotypic antibodies may also be used.

The isolated cytotrophoblast/chorionic villus proteins of the present invention, more preferably the proteins listed in Table 1, may be used as antigens to raise trophoblast or chorionic villus protein specific antibodies. In brief, an immunogen, (the purified trophoblast- or chorionic villus-released protein) is mixed with an adjuvant and animals are immunized with the mixture. The specific amounts will vary in accordance with the protein and the animals used. In general, 1 to 2 mg/kg of body weight are injected and about 1 to 3 doses is common. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Description of techniques for preparing such monoclonal antibodies may be found in, *e.g.*, Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) *Antibodies: A Laboratory Manual* CSH Press; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and particularly in Kohler and Milstein (1975) *Nature* 256: 495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells (*See*, Kohler and Milstein (1976) *Eur. J. Immunol.* 6: 511-519, incorporated herein by reference). The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*.

Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.* (1989) *Science* 246: 1275-1281. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

2) Immunological Binding Assays.

A particular trophoblast- or chorionic villus-released protein can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see *Basic and Clinical Immunology* (7th ed.), Stites and Terr, eds. (1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations (see, *e.g.*, those reviewed in *Enzyme Immunoassay*, Maggio ed. CRC Press, Boca Raton, Florida (1980); Tijan, *Practice and Theory of Enzyme Immunoassays, Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam (1985); and Harlow and Lane *Antibodies, A Laboratory Manual, supra. Immunoassay: A Practical Guide* Academic Press, Chan, ed. Orlando, FL (1987); *Principles and Practice of Immunoassays*, Price and Newman, eds. Stockton Press, N.Y. (1991); and *Non-isotopic Immunoassays*, Ngo ed. Plenum Press, N.Y. (1988); *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); and U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168).

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case

trophoblast- or chorionic villus-released proteins). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds the trophoblast-secreted proteins listed in Table 1.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled trophoblast- or chorionic villus-released protein or a labeled anti-trophoblast- or chorionic villus-released protein antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/trophoblast- or chorionic villus-released protein complex.

In a preferred embodiment, the labeling agent is an antibody that specifically binds to the capture agent (*e.g.*, anti-trophoblast-released protein antibody). Such agents are well known to those of skill in the art, and most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the capture agent is derived. Thus, for example, where the capture agent is a mouse derived anti-human Trophoblast-secreted proteins antibody, the label agent may be a goat anti-mouse IgG; an antibody that is specific to the constant region of the mouse antibody.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species. See, generally Kronval, *et al.*, *J. Immunol.*, 111:1401-1406 (1973), and Akerstrom, *et al.*, *J. Immunol.*, 135:2589-2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

a. Non-competitive assay formats.

Immunoassays for detecting the trophoblast- or chorionic villus-released proteins of the present invention may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case a trophoblast-secreted protein) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (*e.g.*, anti-trophoblast protein antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture a trophoblast-released or chorionic villus-released protein present in the test sample. The protein thus immobilized is then bound by a labeling agent, such as a second human anti-trophoblast-released protein antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived.

b. Competitive assays.

In competitive assays, the amount of analyte (trophoblast-released protein or chorionic villus-released protein) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (*e.g.*, trophoblast-secreted protein) displaced (or competed away) from a capture agent (*e.g.*, anti-trophoblast protein antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, trophoblast-released or chorionic villus-released protein is added to the sample and the sample is then contacted with a capture agent, in this case an antibody that specifically binds the trophoblast-released or chorionic villus-released protein. The amount of trophoblast-released or chorionic villus-released protein bound to the antibody is inversely proportional to the concentration of trophoblast-released or chorionic villus-released protein present in the sample.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of analyte (*e.g.*, trophoblast-secreted protein) bound to the antibody may be determined either by measuring the amount of analyte present in an analyte protein/antibody complex, or alternatively by measuring the amount of remaining uncomplexed analyte protein. The amount of analyte protein may be detected by providing a labeled analyte proteins.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte (*e.g.*, a trophoblast-secreted protein) is immobilized on a solid substrate. A known amount of anti-analyte antibody (*e.g.*, anti-trophoblast-secreted protein antibody) is added to the sample, and the sample is then contacted with the immobilized analyte protein. In this case, the amount of anti-analyte antibody bound to the immobilized analyte protein is inversely proportional to the amount of analyte present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

c. Reduction of non-specific binding.

One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

3) Other assay formats.

Western blot (immunoblot) analysis can also be used to detect and quantify the presence of trophoblast-released or chorionic villus-released proteins in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind trophoblast-released or chorionic villus-released proteins. The anti-analyte antibodies specifically bind to analyte proteins on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.* labeled sheep anti-mouse

antibodies) that specifically bind to the anti-trophoblast-released or anti-chorionic villus-released protein antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)).

B) Detection of Nucleic Acids Encoding Trophoblast-Released or Chorionic Villus-Released Proteins.

Changes in the expression levels of trophoblast-released and/or chorionic villus-released proteins may also be detected by measuring changes in the amount of transcribed mRNA that encodes the trophoblast-released and/or chorionic vullus released proteins. Means of detecting mRNA levels are well known to those of skill in the art. Preferred methods include hybridization and amplification methods.

1) Hybridization methods.

Similarly, a Northern transfer (Northern blot) may be used for the quantification of mRNA encoding trophoblast-released or chorionic villus-released proteins. In brief, the total nucleic acid is isolated from a given cell sample using, for example, an acid guanidinium-phenol-chloroform extraction method and mRNA is isolated by oligo dT column chromatography. The mRNA is then electrophoresed in a suitable electrophoretic gel to separate the mRNA species and the mRNA is transferred from the gel to a nitrocellulose membrane. As with the Southern blots, labeled probes are used to identify the presence or absence of mRNA encoding trophoblast-secreted and/or chorionic villus-released proteins.

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in *Nucleic Acid Hybridization, A Practical Approach*, Ed. Hames, B.D. and Higgins, S.J., IRL Press, (1985); Gall and Pardue, *Proc. Natl. Acad. Sci., U.S.A.*, 63: 378-383 (1969); and John *et al., Nature*, 223: 582-587 (1969).

For example, sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid

in solution. The clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

Typically, labelled signal nucleic acids are used to detect hybridization. Complementary nucleic acids or signal nucleic acids may be labelled by any one of several methods typically used to detect the presence of hybridized polynucleotides. The most common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P-labelled probes or the like. Other labels include ligands which bind to labelled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as a specific binding pair member for a labeled ligand. Labels are discussed in more detail below.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal.

The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or, in some cases, by attachment to a radioactive label. (see, *e.g.*, pp 9-20 in *Practice and Theory of Enzyme Immunoassays*, Laboratory Techniques in Biochemistry and Molecular Biology, Burdon, R.H., van Knippenberg, P.H., Eds., Elsevier (1985))

The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligase chain reaction (LCR) system. Other methods recently described in the art are the nucleic acid sequence based amplification (NASBA, Cangene, Mississauga, Ontario) and Q Beta Replicase systems.

An alternative means for determining the level of expression of a gene encoding a trophoblast secreted protein is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer, *et al.*, *Methods Enzymol.*, 152: 649-660 (1987). In an in situ hybridization assay, cells or tissue

specimens are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to nucleic acids encoding trophoblast-released and/or chorionic villus-released proteins. The probes are preferably labelled with radioisotopes or fluorescent reporters.

2) Amplification methods.

Transcription levels of mRNA may also be quantified by nucleic acid amplification methods such as quantitative polymerase chain reaction (quantitative PCR). Typically quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction.

One preferred internal standard is a synthetic AW106 cRNA. The AW106 cRNA is combined with RNA is isolated from the sample according to standard techniques known to those of skill in the art. The RNA is then reverse transcribed using a reverse transcriptase to provide copy DNA. The cDNA sequences are then amplified using labeled primers. The amplification products are separated, typically by electrophoresis, and the amount of radioactivity (proportional to the amount of amplified product) is determined. The amount of mRNA in the sample is then calculated by comparison with the signal produced by the known AW106 RNA standard. Detailed protocols for quantitative PCR are provided in *PCR Protocols, A Guide to Methods and Applications*, Innis *et al.*, Academic Press, Inc. N.Y., (1990).

C) Labels.

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody or nucleic acid used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays nucleic acid hybridization and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*,

fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions..

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic

labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

D) Solid Supports.

As mentioned above, depending upon the assay, various components, including the nucleic acid probe, antigen, target antibody, or anti-human antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, are included substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium

salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, *J. Biol. Chem.* 245 3059 (1970) which are incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

E. Determination of baseline trophoblast-released and/or chorionic villus-relesed protein levels.

The abnormal maternal-placental interface is detected by a statistically significant alteration in the concentration of one or more of the trophoblast-released and/or chorionic villus-released proteins of the present invention in a particular assay format as compared to the concentration of the same protein determined in the same assay format for a sample from a mammal having a healthy maternal-placental interface.

Healthy, baseline levels of various trophoblast- or chorionic villus-released proteins may be determined by routine screening of samples isolated from mammals having a healthy maternal-placental interface using any of the assay methods described above followed by standard statistical analysis using methods well known to those of skill in the art.

IV. Screening for Compounds that Mitigate Effects of an Abnormal Placental-Maternal Interface.

The *in vitro* model provided by the present invention may be used to screen for therapeutic agents that mitigate the development or effects of an abnormal maternal-placental interface. In one embodiment, these method involve culturing hypoxic trophoblasts or chorionic villi, as described above, in the presence of a potential therapeutic agent and measuring changes in the invasiveness of the trophoblasts. An increase of invasiveness would indicate that the therapeutic agent, may aid in restoration of a normal trophoblast phenotype and hence restoration of normal placental function when utilized *in vivo*.

Methods of quantifying trophoblast invasiveness are described by Librach *et al.*, *J. Cell. Biol.*, 113: 437-449 (1991). Briefly, cytotrophoblasts are plated on Matrigel coated polycarbonate filters. After culture for a defined period of time, the samples are fixed, dehydrated and processed for electron microscopy. Cells penetrating the Matrigel surface can then be quantified from the electron microscope images.

Alternatively, one may screen hypoxic trophoblasts or hypoxic chorionic villi for the effect of potential therapeutic agents on the expression level of the proteins whose expression is altered under hypoxic conditions. It is believed that proteins secreted by the trophoblasts and/or chorionic villi are responsible for numerous maternal complications associated with an abnormal maternal-placental interface. Restoration (*e.g.* downregulation) of normal expression of these proteins will mitigate or eliminate diseases of pregnancy caused by these proteins.

One of skill in the art will appreciate that a wide variety of therapeutic agents may be screened for their ability to block, alter, or otherwise prevent the action of released proteins. Such agents include, but are not limited to, agents that inhibit the activity of the secreted proteins such as metalloproteinase inhibitors or serine protease inhibitors, agents (*e.g.* antibodies, lectins, or other ligands) that bind to and inactivate the TSP or tag the TSP protein for destruction by the immune system. Alternatively, the

agent may act on the expression of the TSP from its respective nucleic acid. Such agents may include suppressor genes, antisense molecules, nucleases, and ribozymes.

IV. Screening for Compounds that Adversely Effect the Maternal-Placental Interface.

Just at there exist agents that mitigate the effects of an abnormal maternal-placental interface, there are other compositions that may adversely effect trophoblast differentiation in a pregnant mammal and thereby induce the formation of an abnormal maternal-placental interface. Clearly it is desirable to identify properties of compounds that may adversely effect development in this manner.

This invention thus provides for methods of screening for agents that might induce the formation of an abnormal maternal-placental interface, or abnormal placental function. In this case, the trophoblasts, or chorionic villi, are cultured under normal conditions and assayed for changes in phenotype as compared to normal and/or hypoxic trophoblasts. Phenotypic changes similar to hypoxic trophoblasts or hypoxic chorionic villi are indicative of possible interference or alteration of the maternal-placental interface by the agent. As described above, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed by the trophoblasts or chorionic villi. Proteins whose expression is typically altered in hypoxic trophoblasts or hypoxic chorionic villi are preferably assayed, while the proteins listed in Table 1 are most preferably assayed.

VII. Kits for the Detection of Abnormal Placental Function

The present invention also provides for kits for the diagnosis of women having an abnormal maternal-placental interface and therefore at risk for diseases of pregnancy such as threatened abortion, intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. The kits preferably include an antibody that specifically binds to one of the trophoblast-released or chorionic villus-released proteins of the present invention or a nucleic acid that specifically hybridizes to another nucleic acid that encodes one or more of the TSPs or chorionic villus-released proteins. The antibody may be free or immobilized on a solid support such as a test tube, a microtiter plate, a dipstick and the like. The kit may also

contain instructional materials teaching the use of the antibody or nucleic acid in an assay for the detection of an abnormal maternal-placental interface.

Additionally, the kit may contain a second antibody that specifically binds the TSP. The second antibody may be labeled, or alternatively, the kit may contain a labeled third antibody that specifically binds the second antibody. The kit may also contain appropriate control series of TSP, buffer solutions, positive and negative controls, washing solutions, dilution buffers and the like for the preparation and analysis of the TSPs in blood or other biological samples..

VIII. Method of Modelng Invasive Cells.

The normal human cell type most closely resembling the phenotype of a cancer cell is the placental trophoblast (Manes, *Cancer Res.*, 34: 2044-2052 (1974)), which shares with malignant cells the ability to invade other tissues, to metastasize, and to evade the body's immune response. Only in rare instances, however do trophoblasts become truly malignant as choriocarcinoma. A wide range of cancer cells secrete hormones and proteins characteristic of trophoblasts, and it has been proposed that malignancy is, in part, a pathological recapitulation of normal placental development (Conway, *J. Theoret. Biol.*, 100: 1-24 (1983)).

Thus, the *in vitro* model of the present invention may also be used as a general model for invasive cells types, in particular for invasive cancer cells. Thus the hypoxic trophoblasts of the present invention may also be used to screen for therapeutic agents that inhibit invasiveness and therefore reduce metastases. As described above, the hypoxic trophoblasts may be used to screen changes in invasiveness or, alternatively, for alterations in the expression of proteins that mediate the invasive activity of the cells.

The hypoxic trophoblasts of the present invention may also be used to identify previously unknown proteins found on cancer, in particular invasive cancer, cells. First, proteins whose expression is altered in hypoxic trophoblasts, are identified as described above. Then cancer cells are screened for the presence of the same or closely related proteins. Methods of screening are well known to those of skill in the art. Preferred methods involve screening for immunologic cross reactivity with antibodies raised against the trophoblast proteins. Alternatively, cancer cell mRNA may be screened for sequences that hybridize to nucleic acid probes complementary to nucleic acid sequences or subsequences that encode the trophoblast secreted proteins.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

Example 1

Culturing Cytotrophoblasts in an Hypoxic Environment Changes Their Antigenic Phenotype to That Seen in Preeclampsia

Highly purified populations of human cytotrophoblasts are isolated as described by Librach *et al., J. Cell. Biol.*, 113: 437-449 (1991). Briefly, cytotrophoblasts are isolated from first, second and third trimester human placentas according to the methods of Fisher, *et al. J. Cell Biol.*, 109: 891-902 (1989), Fisher *et al. Troph. Res.*, 4: 115-138 (1990); and Kliman *et al. Endocrin.*, 118: 1567-1582 (1986), respectively. Enzyme incubation times differ for different lots of collagenase, hyaluronidase, and trypsin. Yields per gram of placentas are about 0.5 - 1.0 x 10^6 cells. Greater than 95% of the cells are cytotrophoblasts, as determined by fluorescent-activated cell sorting.

Remaining leukocytes are removed using an antibody to CD-45, a protein tyrosine phosphatase found on bone marrow-derived cells (Charbonneau *et al. Proc. Nat. Acad. Sci., USA,* 86: 5252-5256 (1989)), but not on cytotrophoblasts. The antibody (Hle; Becton Dickinson and Col, Mountain View, CA, USA, or IgG affinity purified from the GAP 8.3 hybridoma; American Type Culture Collection, Rockville Maryland, USA) is coupled to magnetic beads (Advanced Magnetics Inc., Cambridge, Massachusetts, USA) and mixed with the cytotrophblast-enriched Percoll gradient fraction at a density of 25 particles/cell. After incubation for 20 minutes at 4°C with occasional gentle mixing, the CD-45-positive cells are removed by means of a Bio-Mag Separator (advanced Magnetics, Inc.).

Cytotrophoblasts are then cultured on basement membrane substrates in either a normoxic or a hypoxic (pO_2 = 14 mm Hg) environment. Cells are cultured in conditioned medium (DMEM H21 containing 2% Nutridoma) for 48 hr. It is important to note that serum is not added to the culture medium. Thus, complications in the analysis caused by the introduction of this major source of protein are avoided.

Control cultures are placed in a standard tissue culture incubator containing 8% carbon dioxide and air. When cultured under hypoxic conditions, cells

are placed in sealed culture chambers containing 10% CO_2, 0 to 10% O_2 with the balance being nitrogen.

Differentiation of the normoxic cytotrophoblasts is accompanied by acquisition of an invasive phenotype, as well as by up regulation of the expression of integrins $\alpha5/\beta1$ and $\alpha1/\beta1$, the 92 kDa type IV collagenase and HLA-G. In contrast, hypoxic cytotrophoblasts have a distinctly different antigenic phenotype. They upregulate $\alpha5/\beta1$ expression, showing that they are capable of carrying out at least of a portion of the normal differentiation program. However, they express very low levels of $\alpha1/\beta1$, the 92 kDa type IV collagenase and HLA-G. The cells also fail to invade. This is the exact same antigenic phenotype that characterizes cytotrophoblast differentiation in preeclampsia and indicates that these culture conditions can be used to identify unique proteins synthesized by hypoxic cytotrophoblasts that are candidates for the toxic placental factors that are thought to produce the maternal syndrome.

Example 2

Hypoxic Cytotrophoblasts Up-Regulate their Synthesis of a Discrete Set of Proteins

Cytotrophoblasts, cultured in normoxic and hypoxic conditions, as described in Example 1, are labeled by incorporating in the culture medium 50 μCi/ml [^{35}S]cysteine and [^{35}S]methionine to facilitate the quantitative analysis of newly synthesized proteins. (125 μCi/ml ^{14}C-labeled amino acids (high specific activity ^{14}C-methylated mixture could also be used.)

After 48 h, the medium is dialyzed to remove unincorporated radioactivity. Secreted proteins were precipitated in 80% cold acetone, resuspended in gel loading buffer containing 9 M urea and 2% *n*-octyigiucoside and then stored at -70°C prior to electrophoresis.

The secreted protein products are analyzed by 2-dimensional (2-D) SDS-PAGE and the labeled proteins visualized by autoradiography. Samples were run on analytical scale 2-D gels as originally described by Patton *et al. Biotechniques* 8: 518(1990), with precautions suggested by Hunkapillar *et al. Methods in Enzymology,* 91: 227 (1983). Briefly, 10 μl of sample containing 400,000 dpm are loaded on 1 mm thick first dimension IEF tube gels containing 2.75% ampholytes (2.1, pH 4-8; pH 3-10), and focused under constant voltage to 18,000 volt-hours. Next, the extruded IEF tube gels are loaded on top of 1 mm thick SDS-PAGE slab gels (pH 8.8) and electrophoresed for 5 h under constant power (20 watts/gel). Following electrophoresis, the gels are fixed in

50% methanol containing 10% acetic acid for 12-18 hr, soaked in fluor and exposed to film for various time periods.

Proteins whose expression is altered in hypoxic trophoblasts are listed in Table 1. In three separate experiments, trophoblasts cultured under hypoxia showed at least a fivefold increase in their production of 5 proteins (designated protein A through E in Table 1) ranging in molecular weight from about 21 to about 62 kDa. Additionally, three proteins were identified (proteins E, F and G) whose expression was downregulated in hypoxic trophoblasts.

Example 3

Further Characterization of Proteins Whose Expression is Altered in Hypoxic Cytotrophoblasts

A) Quantitative computer-based analysis of 2D gels.

To determine more precisely the effects of hypoxia, fluorographic exposures of control gels from control and experimental cultures are be visually inspected and computer matched and analyzed. Multiple exposures are scanned with a digital imaging camera interfaced to a SUN3/260 computer. The data are analyzed as previously described (Bersini *et al.*, *Electrophoresis*, 11: 232 (1990)) with the PDQUEST software system, a modification of the software originally developed by Garrels, *Meth. Enzymology*, 100: 411 (1983). This system permits the analysis of 1000-2000 protein spots per gel, depending upon the cell-type and the length of time the gels are exposed to film. These spots may range in abundance from $>10{,}000$ ppm (1%) of total labeled protein (*e.g.*, abundant proteins such as actin) to <10 ppm for the most faint proteins, detected only after a one-month fluorographic exposure. An abundance of 10 ppm corresponds to approximately 10,000 copies of the protein per cell.

Protein spots that are highly resolved and induced in relative abundance of hypoxia are selected for sequencing using the mass spectrometric methods described below.

B) Preparative-scale 2-D gels.

Preparative gels are run to obtain purified preparations containing several hundred picomoles of individual proteins. The methods are similar to those described above for analytical gels with the following modification. To accommodate the loading

of gels with larger amounts of proteins (500-750 μg in 50-150 μl), samples are concentrated severalfold using a Centricon 10 centrifugal concentrator. Isoelectric focusing is done in 3.0 mm internal diameter glass tubes. In the first dimension IEF gels, ampholytes are increased to 5.5% and acrylamide is increased to 4%. Extruded IEF gels are soaked for 30 min. in pH 6.8 equilibration buffer. The second dimension SDS slab gels each contain a 2 cm stacking gel (pH 6.8), and the IEF gels are sealed on top of the SDS slab gels with agarose. Following electrophoresis, gels are stained with Coomassie blue, the protein spots of interest excised with a scalpel, and the gel plugs stored frozen at 20°C until electroelution.

C) Isolation, purification and quantitation of proteins from 2-D gel plugs.

Proteins are isolated from 2-D gel plugs using an electroelution apparatus designed by Hunkapillar (1983), *supra*, or Hoefer Scientific Instruments (San Francisco, California, USA). (Previous studies have demonstrated that more protein can be recovered from polyacrylamide gels by electroelution than by electroblotting onto nitrocellulose or polyvinylidene difluoride membranes.) Electroelution is performed at room temperature and the selected apparatus is fitted with dialysis membrane having the appropriate pore size to insure quantitative recovery of proteins while allowing unwanted impurities to pass. Subsequently, a Konigsberg acetone precipitation (Konigsberg *et al. Meth. Enzymol.*, 91: 254 (1983)) is carried out in order to remove Coomassie blue stain and residual SDS.

D) Proteolytic digestion of proteins and separation of the digest components.

The proteins are enzymatically digested with high purity trypsin (enzyme/substrate ratio = 1:20) in the presence of 4M urea at 37°C. The digestion is carried out for 16 hr to insure complete digestion of the proteins. Separation of the resulting digestion components is achieved on a reserved phase C_{18}, microbore (1.0 mm diameter x 10 cm) HPLC column which, prior to sample injection, is equilibrated with 0.1% trifluroroacetic acid (TFA) in water (Solvent A). Isocratic elution with Solvent A for 10 minutes is followed by a linear gradient (0.5% per min.) to a final mobile phase composition of 70% acetonitrile/30% water/0.1% TFA at a flow rate of 50 μl/min. Peptide elution is monitored at 215 nm and fractions are collected either manually or with an automated fraction collector.

E) Mass spectrometric sequencing of peptides obtained from proteolytic digestion: Molecular weight mapping of separated proteolytic digest components.

Matrix assisted laser desorption ionization (MALDI) is used (Hall *et al.*, *Proc. Nat. Acad. Sci. USA*, 90: 1927 (1993). This highly sensitive technique allows determination of the peptide molecular mass for each HPLC fraction from as little as 1/50 of the entire sample.

Once the molecular weight of each tryptic peptide is determined, high energy collision induced dissociation (CID) mass spectra is determined from the remainder of each HPLC fraction. When some fractions contain multiple components an advantage of tandem mass spectrometric over Edman sequencing is that each component from such a mixture is selected and sequenced in turn. A high performance Kratos Concept IIHH four sector tandem mass spectrometer (Kratos Instruments, Ramsey, New Jersey, USA) equipped with a cesium ion source, continuous flow sample introduction and a scanning, charge-coupled device multichannel array detector is used to obtain the CID mass spectra (Burlingame *et al. Analyt. Chem.*, 66: 634R-683R (1994)).

The first mass spectrometer is used to select the ^{12}C isobar of the protonated molecular ion of the peptide of interest. This species is then be accelerated into a collision cell, floating at 4keV, where it collides with helium atoms. The resulting collisions impart sufficient vibrational energy into the peptide to induce fragmentation. These fragments then pass into the second mass spectrometer where they are mass analyzed and their relative abundances recorded. Complete high energy CID mass spectra are recorded every 11 sec. as sample begins to flow into the ion source from a liquid inlet system containing the peptides of interest. This recently developed unique technology optimizes sensitivity and minimizes low level sample losses such that peptide sequence analysis can be carried out at the subpicomole level. This is achieved using a multi-channel array detection system with a charge-coupled device readout of the spectrum in real time (Burlingame *et al. Analyt. Chem.*, 66: 634R-683R (1994)). The sequence of peptides are deduced with the aid of interactive computer algorithms developed by Hines *et al.*, *J. Am. Soc. Mass. Spectrom.*, 3: 326 (1992).

F) Computer search of Dayhoff and PIR protein data bases for related sequences of known proteins.

Peptide sequences deduced from the interpretation of the CID mass spectra are used to search the Dayhoff and PIR protein data bases in an attempt to relate them to known individual proteins or families of proteins.

F) Confirmation of sequenced proteins induced by hypoxia at the nucleic acid level.

The sequences of the induced proteins may be confirmed by redundant sequencing either by tandem mass spectrometry or, alternatively, the nucleic acid sequences encoding the proteins are cloned and sequenced using standard techniques known to those of skill in the art.

Example 4

Isolation and Sequencing of Proteins Whose Expression is Altered in Hypoxic Chorionic Villi

A) Hypoxia alters chorionic villus release of a discrete number of unlabeled proteins in culture.

Having used two-dimensional electrophoresis (PAGE), as described above, to measure protein synthesis/secretion changes in hypoxic cytototrophoblasts, a similar experiment was attempted with an alternate model system,; explanted chorionic villi. Anchoring chorionic villi, dissected from first trimester placenta were transferred to a mixture of F12 HAM/DMEM (1:1/v:v) culture medium (H/D medium) containing 10% fetal calf serum (FCS). Initially, FCS was included because of the expectation that it might promote the cells' attachment to the substrate. Six villi/well were plated in Matrigel-coated 35-mm culture dishes. One dish (4-6 wells) was placed in an hypoxic atmosphere (2% 0_2) and the other dish was placed in a standard tissue culture incubater (20% O_2). After 24 h, the medium was aspirated and replaced with H/D medium that contained 2% Nutridoma. It was expected that the proteins in Nutridoma would be easily distinguished from those released by the villi in subsequent two-dimensional electrophoresis.

After 72 h, culture medium was collected and centrifuged through a Centricon concentrator with a 10 kDa cutoff. The samples (700 μg protein in 50-150 μL) were subjected to PAGE as described above. After electrophoresis, the gels were

stained with colloidal Coomassie Blue Fast Stain (ZOION), then destained by incubation in methanol/acetic acid/H_2O (45/10/45:v/v/v).

This experiment was repeated four times using villi from different placentas. The results of each were essentially the same. The two-dimensional electrophoresis maps of culture medium samples from cultures maintained under experimental hypoxia and control conditions were nearly identical; the abundance of only a few proteins changed in response to lowering O_2 tension. The results were similar to those shown in Figures 1 and 2 (2D gels for chorionic villi grown in F12 HAM/DMEM, *see* section C, below).

B) Sequencing of proteins whose expression/secretion is altered in hypoxic chorionic villi.

Mass spectrometry (MS) sequencing was used to determine the identity of proteins whose abundance changes in hypoxia. This approach was initially applied to one protein spot whose release into culture media was dramatically increased when chorionic villi were cultured under hypoxic conditions. This spot was excised from six gels and subjected to a 5-step in-gel trypsin digestion as follows:

(1) The gel plugs were macerated to minimize the size of gel particle and to facilitate enzyme access to the trapped protein.

(2) The gel particles were destained by three washes (15 min each) with 25 mM ammonium bicarbonate/50% acetonitrile.

(3) Destained particles were dried for 30 min by vacuum centrifugation (in a Speed-Vac).

(4) The trapped protein was digested by rehydrating the gel particles in 25 mM ammonium bicarbonate buffer containing 0.1 μg/μL high grade trypsin.

(5) Peptides were recovered by three extractions of the digestion mixture with 50% acetonitrile/5% TFA.

Control digestions of gel plugs without protein were performed so that trypsin autoproteolysis products could be identified and disregarded in the MS spectra. A portion (1/25th) of the digest was co-crystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed using a VG TofSpec SE Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometer equipped with a nitrogen laser and

operated in reflectron mode. Digests were analyzed without first being separated by HPLC, thereby saving time and conserving sample. All MALDI spectra were externally calibrated using a standard peptide mixture.

The resulting mass spectra contained two types of information. First a peptide -mass fingerprint was obtained by measuring the monoisotopic molecular masses ($\pm$0.5 Da) of several peptides in the digest. Then peptide sequence information was obtained in the form of a peptide fragment ion tag by acquiring MALDI post-source decay spectra following ion-gating of individual peptides. a peptide fragment ion tag could contain a variety of sequence-ion types produced by several different cleavage processes (a, b, y, a-NH_3, b-NH_3, y-NH_3, b-H_2O, and ammonium ions).

Genomic databases (*e.g.*, GenBank) were searched for known proteins whose peptide-mass fingerprints and peptide sequences matched those in the MALDI spectra of the peptides recovered from the two-dimensional electrophoresis spot.

The results showed the protein was apolipoprotein a-1 (apo a-1). Surprisingly, the protein match was bovine, not human. Evidently the sampled protein came from the medium in which the villi were incubated during the first 24 hours of the 72 hour culture period (H/D with 10% FCS). Nevertheless it was hypothesized that release of the human protein might be similarly regulated.

To determine if this was the case, a mouse monoclonal antibody against human apo a-I was used to analyze, by Western blotting, culture medium samples from chorionic villi maintained under control and experimental hypoxia conditions. In control samples, a band corresponding to the molecular weight of apo a-1 (28 kDa) was barely visible. In contrast, the abundance of this protein was greatly increased in culture medium samples from villi maintained in 2% O_2. a 17 kDa band, which it is believed corresponds to a fragment produced by cleavage in a proteinase-sensitive region of the molecule was also detected. Higher molecular weight diffuse bands were due to a low level of antibody reactitivity with the protein components of Nutridoma. These experiments show that human apo a-I is more abundant in culture media when chorionic villi are cultured under hypoxic conditions.

Using the above-described sequencing methods, other proteins whose expression/secretion levels vary in hypoxic chorionic villi were identified (*see* Table 2). These include, for example, placental lactogen, chorionic gonadotropin, and fibrinogen.

Table 2. Protein expression/secretion in hypoxic chorionic villi.

Spot Number[1]	Change in Hypoxia[2]	Protein Name[3]	Amino acid sequence[4]	Sequence ID NO.
1	down			
2	down	Placental lactogen	LFDHAMLQAHR	1
			ISLLLIESWLEPVR	2
3	down	Placental lactogen	NYGLLYCFR	3
			LFDHAMLQAHR	4
			ISLLLIESWLEPVR	5
			AHQLAIDTYQEFEETYIPK	6
4	up			
5	up	Apolipoprotein a-1	WHEEVEIYR	7
6	down			
7	up	Chorionic gonadotropin	VLQGVLPALPQVVCNYR	8
8	down			
9	unchanged	78 kDa glucose-regulated protein	FELTAIPPAPR	9
			NSLESYAFNMK	10
			DNHLLGTFDLTGIPPAPR	11
10	up	Fibrinogen	LYIDETVNDNIPLNLR	12
			IRPFFPQQ	13
11	down	Serum albumin	RHPEYAVSVLLR	14
			LGEYGFQNALIVR	15
			DAFLGSFLYEYSR	16
			KVPQVSTPTLVEVSR	17
			RPCFSALTPDETYVPK	18
12	down	Serum albumin		
13	down	Serum albumin		
14	down			
15	down			
17	up			
19	unchanged		FELTAIPPAPR	19
			DNHLLGTFDLTGIPPAPR	20

21	up
22	down

1. See, *e.g.*, Figure 1 and Figure 2 for identification of spots. A protein of spot n (where n is the spot number) will appear in the same position relative to the other spots as the spot n in Figure 1 and/or Figure 2.
2. Change in abundance of protein released by hypoxic chorionic villi cultured under 2% O_2.
3. Nominal protein identity as determined by a search of GenBank. It is recognized that while the sequenced fragments share identity with the identified protein, the protein abnormally expressed under hypoxic conditions can also be a fragment, a mutant, a post-translationally modified variant, a species variant, or another variant of the identified protein.
4. Amino acid sequence(s) of tryptic fragments of protein extracted from the spot.
5. The same as spot 11 by peptide mass fingerprint.
6. Identified in hypoxic chorionic villi cultured in FCS.

C) Immunolocalization of apo a-1.

Immunohistochemistry was used to confirm the predicted changes in placental protein level changes in preeclampsia. Accordingly, the antihuman apo a-I antibody was used to stain tissue sections of the placenta and placental bed of preeclamptic (n=5) and control (n=4) patients. Immunolocalization of apo a-I was performed essentially as described by Zhou *et al.* (1993). To identify trophoblasts, the sections were double stained with an antibody that recognized cytokeratin (CK).

In normal pregnancy, the apo a-I antibody reacted with various elements of the floating chorionic villi, including cytotrophoblasts and the syncytial brush border. This observation is consistent with experimental evidence showing that the placenta expresses an apo a-I binding protein (Ehnholm *et al.* (1991)).

Elements of the stromal core were also stained, although they were weak and diffuse. In preeclampsia, placental staining for apo a-I was greatly enhanced in all locations. Within chorionic villi, elements of the stromal core were clearly defined by antibody reactivity. Blood vessels were particularly well outlined. Within the placental bed, cytotrophoblast staining was easily seen.

These experiments show that hypoxic cytotrophoblasts and chorionic villi provide a useful model system for the abnormal maternal/placenta interface. Proteins whose regulation varies with hypoxia and which appear to be good markers for the identification (diagnosis) of the abnormal maternal/placental interface and attendant pathology (*e.g.*, preeclampsia) were identified.

D) Alteration of culture media.

In addition, the results of these experiments suggest that placental cells take up proteins from the medium and these proteins can be later released. Although the described sequencing methods can easily determine whether 2D gel spots are bovine or human proteins, culturing the cells in defined media avoids this complication. Therefore, cell attachment and protein release by chorionic villi cultured in the following treatments:

(1) H/D containing 10% FCS for the first 24 hours, then switched to H/D containing 2% Nutridoma for the last 48 hours (as described above);

(2) H/D with 2% Nutridoma for 72 hours; and

(3) H/D for 72 hours.

These experiments were repeated twice with the same results. Attachment and appearance of the villi did not differ in any of the media. In addition, all three conditions gave essentially the same 2D maps except that the spot which contained bovine apo a-I was missing in samples prepared in medium 2 and Nutridoma protein additives were missing in culture media samples prepared in medium 3.

Therefore, in a preferred embodiment, chorionic villi are cultured in F12 HAM/DMEM (1:1/v:v) -- medium 3. An illustration of two-dimensional electrophoresis gels produced from chorionic villus cultures in medium lacking FCS under normoxic and hypoxic conditions respectively are provided in Figures 1 and 2, respectively.

In summary, the experiments described above indicate that hypoxia changes the repertoire of substances the placenta synthesizes and/or releases. These factors include molecules whose expression is known to be regulated by O_2 tension in other cells (interleukins and growth factors), known proteins whose abundance was not previously known to change in preeclampsia (*e.g.*, apo a-I), and proteins that have yet to be sequenced.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A protein released by a mammalian fetal trophoblast cell or a chorionic villus wherein the level of release is substantially changed when the cell or villus is grown under hypoxic conditions characterized by a partial pressure of oxygen (pO_2) of 14 mm of mercury (mm Hg), wherein said protein is selected from the group of proteins consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2;

(j) A protein of spot number 3 comprising an amino acid sequence selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence sequence 12, and sequence 13 as shown in Table 2;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2; and

(p) A human apolipoprotein A-1.

2. A protein of claim 1, wherein the protein is selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased; and,

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased.

3. A protein of claim 1, wherein the protein is selected from the group consisting of:

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased.

4. A protein of claim 1, wherein the protein is selected from the group consisting of:

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased.

5. A method of culturing human fetal trophoblast cells or chorionic villi under hypoxic conditions, said method comprising the step of culturing the trophoblast cells or chorionic villi under an atmosphere comprising less than about 20% oxygen.

6. A method of claim 5, wherein the method further comprises measuring the release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased.

7. A method of detecting hypoxic cytotrophoblast cells or hypoxic chorionic villi, said method comprising measuring the release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased;

wherein the release of the protein is increased or decreased relative to identical cells grown under identical culture conditions but under normal oxygen conditions.

8. A method of claim 7, wherein the measurement is by direct determination of the protein.

9. A method of claim 7, wherein the determination comprises the step of binding an antibody to the protein and determining the quantity of bound antibody present in a sample relative to the quantity of antibody bound to protein obtained from normoxic trophoblasts or normoxic chorionic villi.

10. A method of claim 7, wherein the determination comprises detecting mRNA encoding any of the proteins and determining if the level of mRNA has changed relative to similarly treated normoxic cells.

11. A method for detecting an abnormal placental function by analysing a biological sample from a pregnant mammal for abnormal release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and,

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15,

sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased..

12. A method of claim 11, wherein said abnormal placental function is a symptom of a disease of pregnancy selected from the group consisting of threatened abortion, intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia.

13. A method of claim 12, wherein said disease of pregnancy is preeclampsia.

14. A method of screening for agents that mitigate the effects of an abnormal maternal-placental interface, said method comprising:

(i) culturing cytotrophoblasts under hypoxic conditions in the presence of said agent; and

(ii) assaying for changes in the phenotype of said hypoxic trophoblasts relative to hypoxic trophoblasts cultures without the presence of said agent.

15. The method of claim 14, wherein said assaying comprises measuring the invasiveness of said trophoblasts.

16. The method of claim 14, wherein said assaying comprises measuring the changes in the levels of release of proteins expressed by said trophoblasts.

17. The method of modeling, *in vitro*, an abnormal maternal-placental interface, said method comprising culturing trophoblast cells or chorionic villi in a hypoxic environment.

18. The method of claim 17, wherein said hypoxic environment comprises an atmosphere comprising less than about 20% oxygen.

19. A method for identifying proteins that are indicative of metastasis said method comprising:

(i) raising cytotrophoblasts under hypoxic conditions; and

(ii) detecting proteins that demonstrate an altered release level as a result of said hypoxic conditions; and,

(iii) determining if said proteins are present in metastatic cells.

20. A method of claim 19, wherein the determining is done by immunoassay using antibodies specific for at least one of the proteins of step ii.

21. A method for identifying proteins that are indicative of an abnormal maternal placental interface said method comprising:

(i) culturing cytotrophoblasts under hypoxic conditions; and

(ii) detecting proteins that demonstrate an altered release level as a result of the hypoxic conditions.

22. A method of claim 21, wherein said abnormal maternal placental interface is indicative of preeclampsia.

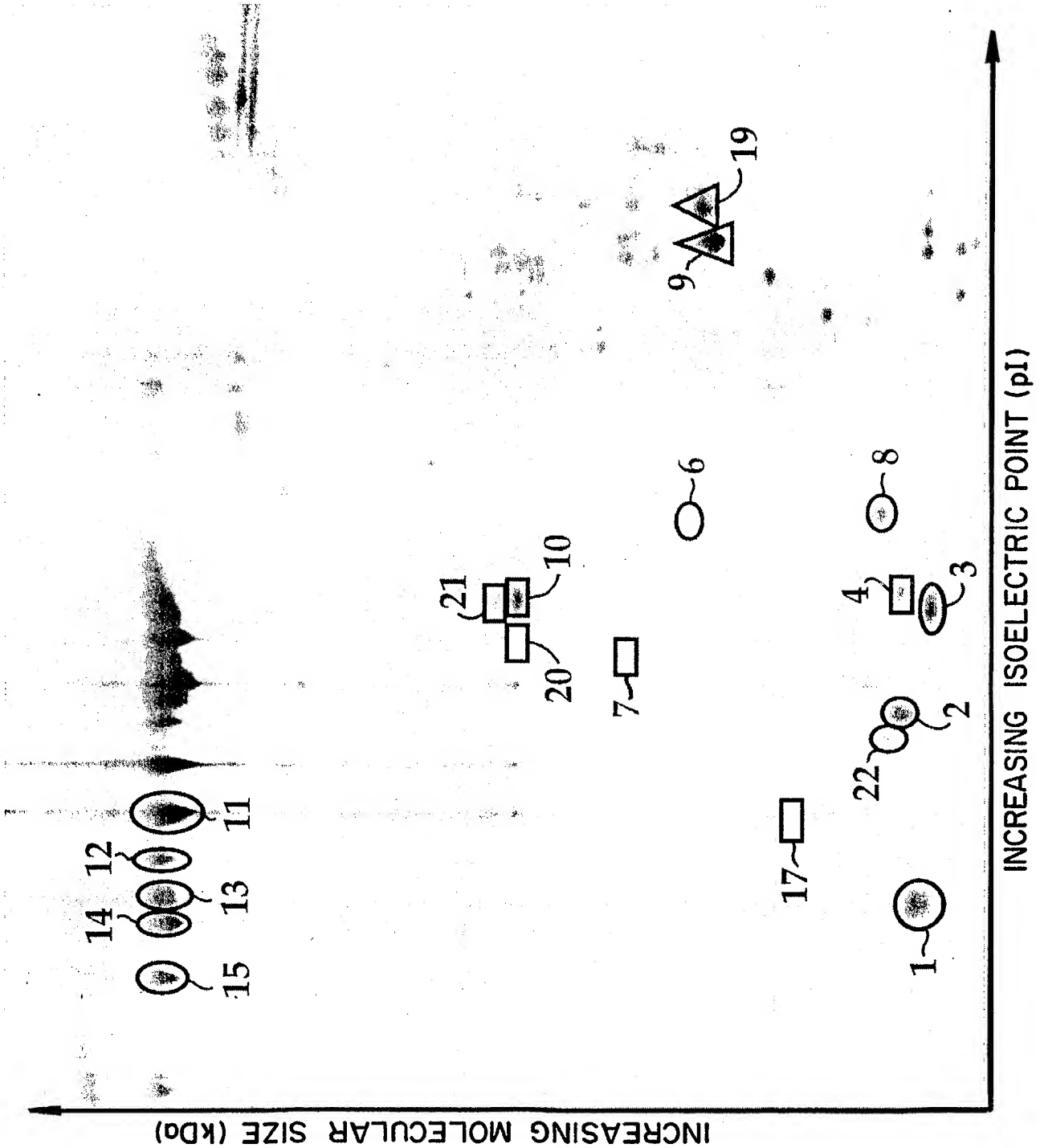
INCREASING MOLECULAR SIZE (kDa)
INCREASING ISOELECTRIC POINT (pI)
1
2
3
4
6
7
8
9
10
11
12
13
14
15
17
19
20
21
22
DOWN in hypoxia
UP in hypoxia
unchanged

FIG. 1.

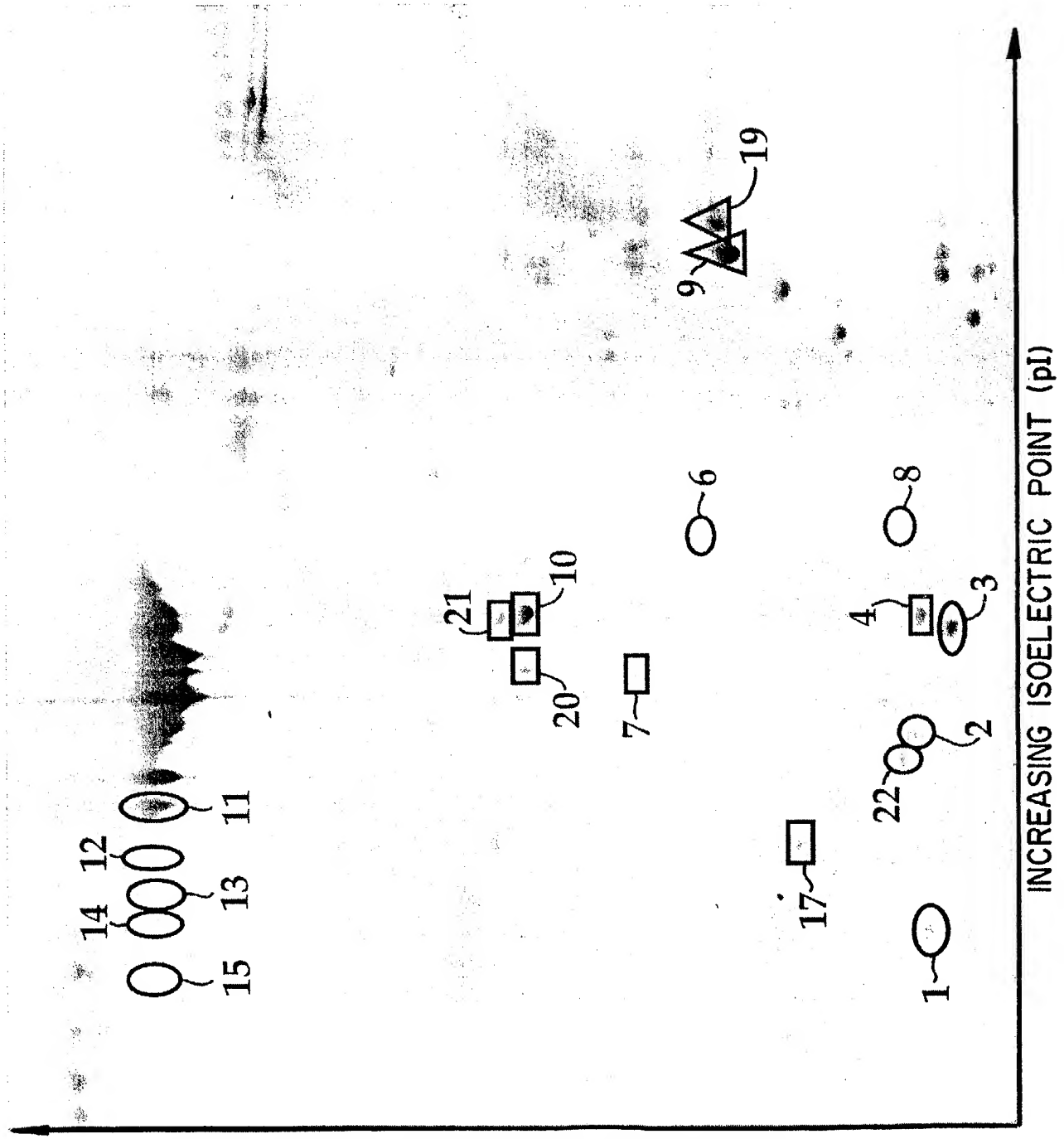
INCREASING MOLECULAR WEIGHT (kDa)
INCREASING ISOELECTRIC POINT (pI)
1
2
3
4
6
7
8
9
10
11
12
13
14
15
17
19
20
21
22
DOWN in hypoxia
UP in hypoxia
unchanged

FIG. 2.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as designated Office

Date of mailing:
27 December 1996 (27.12.96)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/US96/05441	WO96/33214

CORRECTED VERSION
VERSION CORRIGEE

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 730.91.11

20

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2307E-6001PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 96/05441	18/04/1996	18/04/1995
International Patent Classification (IPC) or national classification and IPC		
C07K14/47		
Applicant		
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of _____ sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/10/1996	10.07.97
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	A. Chakravarty Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.
PCT/US96/05441

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[x] the international application as originally filed.

[ ] the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____,

[ ] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 5,6,14-22	YES
	Claims 1-4,7-13	NO
Inventive Step (IS)	Claims 5,6-14-22	YES
	Claims 1-4,7-13	NO
Industrial Applicability (IA)	Claims 1-22	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

1. Reference is made to the following documents:

D1 = J. CLIN. INVEST. (1996), 97(2), 540-550
D2 = US-A-5 141 849
D3 = Derwent Abstract, accession number 82-13463E & SU-A-827030
D4 = WO-A-93/24 838
D5 = US-A-5 196 523
D6 = US-A-4 943 527

Novelty

2. The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of Claims 1-4 and 7-13 is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).
Claims 1-4 are product claims addressed to proteins per se. The fact that the level of expression of these proteins alters in hypoxic foetal trophoblasts cannot serve to establish novelty.

D2 (columns 1 and 2) discloses pregnancy specific ß1-glycoprotein (72, 64 and 54kD) as being associated with foetal hypoxia and preeclampsia.
Since molecular weights must be seen as including an error of approx. +/- 10%, this disclosure anticipates claims 1-3 and 7-13.

D3 discloses placental lactogen and its use in diagnosis of foetal hypoxia, anticipating the subject-matter of claims 1-4 and 7-13.

D4 discloses chorionic gonadotropin and its use in diagnosis of preterm delivery, anticipating the subject-matter of claims 1-4 and 11-13.

D5 discloses 78kD glucose regulated protein and thus anticipates the subject-matter of claims 1-4.

D6 discloses APO-A1, anticipating the subject-matter of claims 1-4.

Inventive Step (Article 33(3) PCT and Rule 65(1)(2) PCT)

3. The following comments concern the remaining, novel subject-matter only.
The closest prior art to the present subject-matter is regarded as being D2 and D3.
In the light of the prior art and having regard to the present description and claims, the essential problem underlying the present application can be formulated as either "providing a model for abnormal placental function" (expressly set out in claims 5-6) or "providing a method for detecting abnormal placental function" (claims 7-13).
Neither D2 nor D3 suggests that trophoblast cells cultured under hypoxic conditions might provide a model for the abnormal placenta.
Thus, inventive step can be recognised for the

subject-matter of claims 5-6 and 14-22.
However, use of altered protein levels as an indicator of abnormal placental function was known from D2 and D3 and no inventive step can be recognised for the subject-matter of claims 7-13.

4. The priority documents pertaining to the present application were not available at the time of establishing this Report. Hence, it is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. If it later turns out that this is not correct, the document D1 cited in the international search report could become relevant to assess whether the claims satisfy the criteria set forth in Article 33(1) PCT.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D6 are not mentioned in the description, nor are these documents identified therein.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1. The application does not meet the requirements of Article 6 PCT because the Claims are not clear.

1.1 The definitions of the proteins (a)-(h) in claims 1, 2, 3, 6, 7 and 11, in which the proteins are defined only by their molecular weight and their pI are inadequate to unequivocally identify them. Only those proteins whose definition also includes a sequence are regarded as being adequately defined.

1.2 For the sake of clarity, the amino acid sequence represented by the various sequence I.D.s should be introduced into the claims.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 24 October 1996 (24.10.96)	
International application No.: PCT/US96/05441	Applicant's or agent's file reference: 2307E-6001PC
International filing date: 18 April 1996 (18.04.96)	Priority date: 18 April 1995 (18.04.95)
Applicant: FISHER, Susan, J. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

07 October 1996 (07.10.96)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer: J. Zahra
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 730.91.11

Copy for the Elected Office (EO/US) PCT/US96/05441

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

HUNTER, Tom
Townsend and Townsend and Crew LLP
8th Floor
Two Embarcadero Center
San Francisco, CA 94111
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
22 October 1996 (22.10.96)

Applicant's or agent's file reference
2307E-6001PC

IMPORTANT NOTIFICATION

International application No.
PCT/US96/05441

International filing date (day/month/year)
18 April 1996 (18.04.96)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
HUNTER, Tom
Townsend and Townsend and Crew
Steuart Street Tower
One Market
San Francisco, CA 94105-1492
United States of America

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
HUNTER, Tom
Townsend and Townsend and Crew LLP
8th Floor
Two Embarcadero Center
San Francisco, CA 94111
United States of America

State of Nationality | State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[X] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Ingrid Hours

Telephone No.: (41-22) 730.91.11

Form PCT/IB/306 (March 1994) 001265258

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
2307E-6001PC		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US96/05441	18/04/96	18/04/95
Applicant		
THE REGENTS OF THE UNIVERSITY OF CALIFORNIA et al.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [ ] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [ ] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [ ] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
[redacted]T/US 96/05441

A. CLASSIFICATION OF SUBJEC[redacted]TER
IPC 6 C07K14/47 C07K14/775 C12N5/08 G01N33/68 G01N33/574
G01N33/92

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 141 849 (CHOU JANICE) 25 August 1992 see the whole document ---	1-3,7-13
X	DATABASE WPI Section Ch, Week 8207 Derwent Publications Ltd., London, GB; Class B04, AN 82-13463E XP002011721 & SU,A,827 030 (MOSC MED STOMATOLOG) , 7 May 1981 see abstract ---	1-4,7-13
X	WO,A,93 24838 (ADEZA BIOMEDICAL CORP) 9 December 1993 see the whole document --- -/--	1-4, 11-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1996	23. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chakravarty, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 943 527 (PROTTER ANDREW A ET AL) 24 July 1990 see the whole document ---	1-4
X	US,A,5 196 523 (LEE A.S.) 23 March 1993 see the whole document ---	1-4
X,P	J. CLIN. INVEST, vol. 97, no. 2, 15 January 1996, NEW YORK, NY; US , pages 540-550, XP000579303 GENBACEV O ET AL.: "Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia" see the whole document -----	1-22

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/05441

[redacted]mation on patent family members

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5141849	25-08-92	NONE	
WO-A-9324838	09-12-93	NONE	
US-A-4943527	24-07-90	AU-B- 6522486	24-04-87
		EP-A- 0239631	07-10-87
		JP-T- 63501764	21-07-88
		WO-A- 8702062	09-04-87
US-A-5196523	23-03-93	NONE	

PCT WORLD INTELLECTUAL PROPERTY ORGANI
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/47, 14/775, C12N 5/08, G01N 33/68, 33/574, 33/92

A2

(11) International Publication Number: **WO 96/33214**

(43) International Publication Date: 24 October 1996 (24.10.96)

(21) International Application Number: PCT/US96/05441

(22) International Filing Date: 18 April 1996 (18.04.96)

(30) Priority Data:
08/423,409 18 April 1995 (18.04.95) US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, Oakland, CA 94612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FISHER, Susan, J. [US/US]; 1347 4th Avenue, San Francisco, CA 94122 (US). GENBACEV, Olga [YU/US]; 1050 Crestview Drive #45G, Mountain View, CA 94040 (US). FOULK, Russell [US/US]; 740 Alta Vista, Pacifica, CA 94044 (US). CLAUSER, Karl, R. [US/US]; 3200 Treetops Circle, San Bruno, CA 94066 (US). BURLINGAME, Alama, L. [US/US]; 26 Alexander Avenue, Sausalito, CA 94965 (US).

(74) Agents: HUNTER, Tom et al.; Townsend and Townsend and Crew, Steuart Street Tower, One Market, San Francisco, CA 94105-1492 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

(57) Abstract

This invention is the discovery that hypoxic mammalian fetal trophoblast cells are a valid model of abnormal maternal plancental function and of other invasive cell types. The trophoblast cells differentially express a number of proteins when grown under hypoxic conditions. These proteins are useful for determining the health of the trophoblasts, the condition of a maternal placental interface and the invasive properties of metastatic and other invasive cell types. The hypoxic cytotrophoblast cells provide a convenient means to isolate these proteins and develope assay kits based upon the presence or absence of the proteins in a human test sample.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/566, 33/567, 33/53 C12Q 1/00

A1

(11) International Publication Number: WO 93/24838

(43) International Publication Date: 9 December 1993 (09.12.93)

(21) International Application Number: PCT/US93/04735

(22) International Filing Date: 21 May 1993 (21.05.93)

(30) Priority data:
891,366 29 May 1992 (29.05.92) US

(71) Applicant: ADEZA BIOMEDICAL CORPORATION [US/US]; 1240 Elko Drive, Sunnyvale, CA 94089 (US).

(72) Inventors: SENYEI, Andrew, E. ; 30551 Hilltop Way, San Juan Capistrano, CA 92675 (US). CASAL, David, C. ; 22441 Franklin Court, Mountain View, CA 94040 (US).

(74) Agents: TERLIZZI, Laura et al.; Skjerven, Morrill, MacPherson, Franklin & Friel, 25 Metro Drive, Suite 700, San Jose, CA 95110 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: SCREENING METHOD FOR IDENTIFYING WOMEN AT INCREASED RISK FOR PRETERM DELIVERY

(57) Abstract

The present invention provides an early, biochemical indication of increased risk of preterm delivery. The method comprises obtaining a body fluid sample from a pregnant patient after about week 4 of gestation and determining the proportion of total human chorionic gonadotropin (hCG) in the sample that is in the intact form. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

SCREENING METHOD FOR IDENTIFYING WOMEN AT INCREASED RISK FOR PRETERM DELIVERY

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to methods for detection of increased risk of preterm delivery. In particular, this invention is directed to determining an early indication of increased risk of preterm delivery by determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form in a body fluid sample.

Description of the Prior Art

Determination of impending preterm births is critical for increasing neonatal survival of preterm infants. In particular, preterm neonates account for more than half, and maybe as much as three-quarters of the morbidity and mortality of newborns without congenital anomalies. Although tocolytic agents which can delay delivery were introduced 20 to 30 years ago, there has been only a minor decrease in the incidence of preterm delivery. It has been postulated that the failure to observe a larger reduction in the incidence of preterm births is due to errors in the diagnosis of preterm labor and to the patients' conditions being too advanced for tocolytic agents to successfully delay the birth.

Traditional methods of diagnosis of preterm labor have high false-negative and false-positive error rates [Friedman et al, *Am. J. Obstet. Gynecol.* 104:544 (1969)]. In addition, traditional methods for determining impending preterm delivery, particularly in patients with clinically intact membranes, may require subjective interpretation, may require sophisticated training or equipment [Garl et al, *Obstet. Gynecol.*

60:297 (1982)] or may be invasive [Atlay et al., *Am. J. Obstet. Gynecol.* **108**:933 (1970)]. Accordingly, an early, objective biochemical marker indicative of increased risk for preterm delivery was sought.

Recently, Lockwood et al [*New Engl. J. Med.* **325**:669-674 (1991)] reported that fetal fibronectin in cervical and vaginal secretions indicates pregnancies which are at risk of imminent delivery. The authors postulate that damage to the fetal membranes may release fetal fibronectin into the cervix and vagina, thus giving rise to the biochemical marker.

Other markers which may be released in women with true threatened pregnancies can be used to screen those women who should be closely monitored and to provide additional information about the stage of the disease.

SUMMARY OF THE INVENTION

The present invention provides an early, biochemical indication of increased risk of preterm delivery. The method comprises obtaining a body fluid sample from a pregnant patient after about week 4 of gestation and determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form in the sample. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery. The test is preferably administered to women at about 4 weeks gestation and repeated at each prenatal visit (every two to four weeks) until at least week 37, preferably until delivery if the test is negative. For those patients whose assay result indicates an increased risk of preterm delivery, a test of the patient's fetal fibronectin level can be made to confirm the increased risk and to estimate how soon the delivery will be. In addition, those patients can be carefully monitored, as for other patients at risk.

The test is both a sensitive and specific screen for

pregnancies at risk and can detect an increased risk of preterm delivery as early as two to four weeks prior to delivery. The method not only allows early intervention in the course of preterm delivery but also provides an additional factor which can indicate those pregnancies at greatest risk.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a screening assay which provides an early, biochemical indication of increased risk of preterm delivery based on detection of a decreased proportion of intact human chorionic gonadotropin (hCG) in a body fluid sample. The method can provide an indication of impending delivery as early as two to three weeks prior to delivery. This method allows early intervention in the course of preterm delivery and provides an additional factor which can indicate those pregnancies at greatest risk.

The method comprises obtaining a body fluid sample, preferably serum or urine, from a pregnant patient after about week 4 of pregnancy and prior to about week 36 or 37, and determining the proportion of human chorionic gonadotropin (hCG) that is in the intact form. A decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicates a patient who is at risk for preterm delivery. In a preferred embodiment, the proportion of hCG that is in the intact form is determined using an immunoassay. Since inflammatory conditions in the local membranes could damage trophoblast secreted proteins such as hCG, the amount of intact hCG in the local area as reflected in cervicovaginal secretion samples, as well as the amount systemically (e.g. in blood and urine samples) can be used as an indicator of the damage at the maternal fetal interface.

The present invention can determine increased risk of preterm delivery between weeks 4 and 37 of gestation.

Deliveries between 4 to 20 weeks gestation are generally termed spontaneous abortions rather than preterm deliveries. Term pregnancies are from 37 to 40 weeks.

Intact and Nicked Human Chorionic Gonadotropin (hCG)

The present invention is based on detection of a significant decrease in the proportion of intact hCG that occurs in body fluids of women who deliver preterm. Total hCG is the sum of nicked hCG and intact hCG. The proportion of intact hCG can be determined using any method that determines the relative amounts of any two of total hCG, nicked hCG, and intact hCG.

hCG is a glycoprotein hormone produced almost exclusively by the placenta. The polypeptide portion of hCG is a dimer that is composed of an alpha subunit (92 amino acid residues) and a beta subunit (145 amino acid residues), joined noncovalently. The beta chain contains a disulfide bridge between cysteines 38 and 57. The appearance of hCG in patient urine is currently the most commonly used indicator to determine pregnancy.

hCG is found in the blood and urine of pregnant women as a mixture of two forms: an intact form and a proteolytically nicked form. The nicked form is the same as the intact form but for a single break in the beta-subunit polypeptide chain between either residues 44 and 45, residues 47 and 48, or far less commonly, residues 46 and 47. The nicked form comprises, on average, about one quarter of the total hCG population in both the blood and urine of pregnant women who deliver at term.

The alpha- and beta-chains of hCG are also present as free subunits. In addition, a fragment of the beta-chain, called the beta-core fragment, which comprises beta-chain residues 6-40 disulfide-linked to beta-chain residues 55-92, may also be present.

Other known variants of hCG include truncated forms which lack the first two or three N-terminal amino acids of the alpha chain. However, these truncated forms are

characteristic of relatively rare pregnancy-associated cancers (hydatidiform mole and choriocarcinoma) and are not observed in normal pregnancies.

Patients to be Tested

The present method can be used on any pregnant woman following about 4 weeks gestation and prior to term (week 36 or 37). In addition to screening any pregnant woman to determine whether the patient is at risk for preterm delivery, the patients who are preferably screened are those patients with clinically intact membranes in a high risk category for preterm delivery, and more preferably, all those women whose pregnancies are not sufficiently advanced to ensure delivery of a healthy fetus. Ninety percent of the fetal morbidity and 100 percent of the fetal mortality associated with preterm delivery is for those fetuses delivered prior to 32 to 34 weeks gestation. Therefore, 32 to 34 weeks gestation is an important cutoff for the health of the fetus, and preferably women whose pregnancies are at least about 4 weeks and prior to 34 weeks in gestation are tested.

In addition there are a large number of factors known to be associated with the risk of preterm delivery. Those factors include multiple fetus gestations, incomplete cervix, uterine anomalies, polyhydramnios, nulliparity, previous preterm rupture of membranes or preterm labor, preeclampsia, first trimester vaginal bleeding, little or no antenatal care, and symptoms such as abdominal pain, low backache, passage of cervical mucus, and contractions. Any pregnant woman at 4 or more weeks gestation with clinically intact membranes and having one or more risk factors for preterm delivery is preferably tested throughout the risk period; i.e., until about week 34 to 37.

The Sample

The sample is a body fluid sample, preferably blood,

urine or cervic aginal secretions, and is llected according to standard procedures. A blood sample can be a plasma or, preferably, a serum sample. The sample is preferably frozen following processing if the sample cannot be analyzed within a few hours of collection. The urine sample can be a random sample, preferably a first morning specimen, or more preferably, a 24 hour sample. Urine samples which are not assayed within 24 hours of collection are preferably stored at 4°C, and more preferably, are stored frozen. A cervicovaginal secretion sample is generally obtained from the vaginal cavity or the external cervical canal using a swab having an absorbent material; e.g., cotton or dacron.

Assay Procedure

As stated previously, the proportion of intact hCG can be determined using any method that determines the relative amounts of any two of total hCG, nicked hCG, and intact hCG. Immunoassays that quantitate total and intact hCG are preferred. However, non-quantitative assays that determine relative amounts of nicked and intact hCG or total and intact hCG in a given volume of sample can also be used.

Total hCG is the sum of the nicked and intact forms of hCG. The proportion of intact hCG is the amount of intact hCG divided by the amount of total hCG. Therefore, when the proportion of hCG that is nicked is known, the proportion that is intact is the difference of the total hCG minus the nicked proportion. Similarly, when the relative levels of nicked and intact hCG are known, the proportion of hCG that is intact is the level of intact hCG divided by the sum of the levels of the intact and nicked forms. Therefore, determining the proportion of nicked hCG is equivalent to determining the proportion of intact hCG. Preferably, the proportion of intact hCG is reported as a percentage.

The proportion of intact hCG is preferably determined

by quantitating intact hCG and total hCG using an immunoassay. Alternatively, assays that determine the relative amounts of proteins in a sample, such as Western blot assays, can be used to determine the relative amounts of intact hCG and nicked hCG to determine the proportion of intact hCG. Determining the proportion of total hCG that is in the intact form requires not only distinguishing nicked from intact hCG, but also distinguishing nicked and intact hCG from the free alpha- and free beta-chains.

Antibodies specific for hCG and its subunit chains are well known. At least two epitopes have been reported on the alpha-chain and at least three on the beta-chain. In addition to the epitopes on each of the chains, there is an epitope that is present on the intact hCG dimer which is not present on either of the free chains or on nicked hCG. See, for example, Krichevsky et al [*Endocrinology* **123**:584-593 (1988)] and the references cited therein, which describe various hCG epitopes. That article and the references cited therein are hereby incorporated by reference in their entireties.

Anti-hCG antibodies can be polyclonal or monoclonal for the purposes of the present invention and can be produced and purified by conventional methods. Such methods are described in a number of publications including Tijssen, P. Laboratory Techniques in Biochemistry and Molecular Biology: Practice and Theories of Enzyme Immunoassays New York: Elsevier (1985).

In addition, antibodies to hCG are available. For example, a polyclonal antibody specific for the beta-chain is commercially available (Bios Pacific, Inc., Emeryville, CA). Anti-alpha-chain antibodies are available from Unipath (Cambridge, U.K.). In addition, a monoclonal antibody, designated B109, that is specific for the hCG dimer [described in Krichevsky et al, *Endocrinology* **123**:584-593 (1988); Cole et al, *Endocrinology*

129:1559-1567 (1991)] is used at numerous centers in the U.S. to detect low levels of hCG. This antibody does not recognizes free alpha- or free beta-chains or nicked hCG and, therefore, can be used to distinguish intact hCG from nicked hCG.

At present, there is no known antibody specific for the nicked form of hCG. However, if an epitope is identified that is present on the nicked form that is not present on the intact form, the immunoassay can quantitate nicked hCG and total hCG or quantitate nicked hCG and intact hCG to determine the proportion of intact hCG in the sample.

A number of different types of immunoassays are well known using a variety of protocols and labels. The assay conditions and reagents may be any of a variety found in the prior art. The assay can be heterogeneous or homogeneous and is conveniently a sandwich assay.

The assay usually employs solid phase-affixed antibodies. The solid phase-affixed antibodies are combined with the sample. Binding between the antibodies and sample can be determined in a number of ways. Complex formation can be determined by use of soluble antibodies. The soluble antibodies can be labeled directly or can be detected using labeled second antibodies specific for the species of the soluble antibodies. Various labels include radionuclides, enzymes, fluorescers, colloidal metals or the like. Conveniently, the assay will be a quantitative enzyme-linked immunosorbent assay (ELISA) in which antibodies specific for hCG are used as the solid phase-affixed antibodies and enzyme-labeled, soluble antibodies.

To assay total hCG, the assay can use any two hCG antibodies for hCG that do not compete for a binding site, with the exception of dimer-specific antibodies. Preferably, the assay uses an antibody for the alpha-chain and an antibody for the beta-chain to avoid potential quantitation of either of the free chains. However, when

the relative amount of the free chains is not a significant proportion of total hCG, the assay can use two antibodies to the same chain. To assay intact hCG, the assay can use a dimer-specific antibody together with an antibody for the alpha-chain or, preferably, the beta-chain.

Alternatively, the assay can be based on competitive inhibition, where analyte in the sample competes with a known amount of analyte or analyte analog for a predetermined amount of anti-analyte antibody. In the competitive format, a dimer-specific antibody is used to detect intact hCG and an antibody for either the alpha-chain or the beta-chain is used to quantitate total hCG. When the relative amount of the free chains are not a significant proportion of the total hCG, the free chains do not affect the assay result. However, when the relative amounts of the free chains are significant, preferably an assay using an antibody for the alpha-chain and an antibody for the beta-chain such as a sandwich assay, rather than competitive assay, is used.

A standard with a known amount of intact hCG is used. Preferably, the hCG in the standard is at least 80% intact. Standards with known amounts of intact hCG are available commercially. As recently reported, the Columbia anti-hCG test that has been used to quantitate total hCG in some hCG standards does not detect nicked hCG [Birken et al, Endocrinology 129:1551-1558 (1991); Cole et al, *Endocrinology* 129:1559-1567 (1991)]. Therefore, the hCG standard is preferably one in which the 8proportion of intact hCG has been determined by amino acid sequence analysis.

Other quantitative methods to determine the proportion of hCG that is in the intact form can be envisaged. For example, a method to quantitate total and nicked hCG using amino acid sequence analysis of hCG purified from urine has been described [Khardana et al, *Endocrinology* 129:1541-1550 (1991)]. An exemplary

procedure to determine the proportion of hCG that is intact in a serum or urine sample by quantitating total and intact hCG is described in detail in the Examples.

In addition to methods that quantitate hCG forms, any method that determines relative amounts of total and intact hCG can also be used. For example, a Western blot assay can be used to determine the relative amounts of total and intact hCG by determining the relative amounts of nicked and intact hCG beta-chains. Briefly, the electrophoresis is performed under denaturing conditions which separate the alpha-chain from the beta-chain. A reducing agent, for example β-mercaptoethanol, is used to reduce disulfide bonds in the beta-chains. Following treatment with a reducing agent, the beta-chain of intact hCG remains a single chain with an apparent molecular weight of about 34 to 37 kD and the beta-chain of nicked hCG separates into an N-terminal fragment of about 17 kD and a C-terminal fragment of about 24 kD, as determined by electrophoresis.

Following electrophoresis, the separated proteins are transferred to a support membrane and detected with labeled anti-beta C-terminus antibody. The antibodies react with the intact beta-chain (34-37 kD) and the nicked beta-chain C-terminal fragment (24 kD). The size differences between the intact and nicked chains facilitate distinguishing the intact and nicked beta-chains. The relative amounts of intact and nicked beta-chain are determined by appropriate methods, depending on the label on the antibody. The proportion of hCG in the intact form is the relative amount of intact beta-chain divided by the sum of intact beta-chain and nicked beta-chain C-terminal fragment. Preferably, the original sample is also tested for free, intact beta-chain to ensure that the level of intact hCG is not overestimated.

A Western blot method to determine the proportion of hCG that is in the intact form is described in Cole et al

[*supra*]. Other methods which measure relative amounts of total, nicked, and intact hCG can also be envisaged.

Interpretation of Result

A decrease in the proportion of hCG that is in the intact form relative to the proportion that is characteristic of pregnancies that proceed to term indicates an increased risk of preterm delivery. Preferably, the threshold value that separates risk from non-risk cases is two standard deviations below the average value for pregnancies that proceed to term. A preferred threshold below which the patient is considered to be at risk for preterm deliver is 25% or less intact hCG (as a fraction of total hCG in the sample). A more preferred value is 10% or less intact hCG. As is well known, the 25% threshold value will detect more false positive values. However, a somewhat high false positive rate is acceptable in a screening assay, where the objective is to detect all those at risk. For an assay which has a lower false positive rate, but which has a higher false negative rate, the lower threshold is selected. Since the proportion of intact hCG can also be expressed as the proportion of nicked hCG, or as the ratio of nicked to intact hCG, thresholds appropriate for such expressions can also be used. However, since the proportion of total hCG that is in the intact form varies considerably among women with pregnancies that proceed to term, it is preferable that patients with samples near the threshold value be retested in a follow up visit.

If the hCG test is positive (i.e., the proportion of hCG in the intact form is below the threshold value), the patient is preferably tested for the presence of fetal fibronectin in her cervicovaginal secretions. If fetal fibronectin is present in the secretions, the patient is likely to deliver in two to three days. Measures to determine or enhance fetal lung maturity can be undertaken. If the fetal fibronectin assay is negative,

the patient should be carefully monitored and repeated evaluations of the patient's fetal fibronectin levels should be performed on subsequent visits. In general, patients at risk for preterm delivery are examined every two weeks from about 22 to 36 weeks, rather than every four weeks as for patients in a low risk category.

If the hCG test is negative (the proportion of hCG in the intact form is above the threshold), the test is preferably repeated on each subsequent visit until either the test is positive or the patient reaches term.

The procedure is sensitive and specific. Since the test successfully detects a large percentage of patients who deliver early, the test is an effective screening procedure for women at risk for preterm delivery who do not have any other risk indicators.

This invention is further illustrated by the following specific but non-limiting examples. Temperatures are given in degrees Centigrade and concentrations as weight percent unless otherwise specified. Procedures which are constructively reduced to practice are described in the present tense, and procedures which have been carried out in the laboratory are set forth in the past tense.

EXAMPLE 1

The following immunoassay method determines the proportion of total hCG that is in the intact form. The method can be used for a serum or urine sample. The proportion of total hCG that is intact is determined by measuring the concentrations of intact hCG and total hCG by separate assays.

The concentration of intact hCG in the sample is determined using a B109:anti-β-peroxidase assay performed as described by Cole et al [*Endocrinology* 129:1559-1567 (1991)]. This immunoassay uses a hCG dimer-specific monoclonal antibody designated B109 (available from Drs. A. Krichevsky and E. Armstrong of Columbia University) as

the solid phase-affixed antibody to capture intact hCG, and peroxidase-labeled, goat anti-beta-chain antiserum (Bios Pacific, Inc., Emeryville, CA) to detect bound intact hCG. Microtiter plates are coated with antibody B109 (200 µl; 2 µg/ml in 0.25 M sodium carbonate, pH 9.5, containing 0.1 M NaCl) and plates are incubated overnight at 4°C. Plates are washed five times with water and aspirated before use.

In triplicate, sample or purified, intact hCG standard (100 µl) is added to coated wells together with buffer-carrier protein mix (100 µl; 0.05 M sodium phosphate, pH 7.5, containing 0.14 M NaCl and 0.1% ovalbumin). hCG standard solutions (0, 2.5, 5, 10, 15, 20, and 25 ng intact hCG/ml) are used to establish a standard curve.

The plates are shaken on a plate rotator overnight at ambient temperature and then washed five times with water and aspirated. Peroxidase-labeled goat anti-beta-chain antiserum (200 µl; 1:3500 dilution in 0.1 M Tris-HCl, pH 7.5, containing 0.025 M $CaCl_2$ and 0.1% ovalbumin) is then added to the wells, and the plates are shaken for 2 hours at ambient temperature. After another five washes with water, 200 µl of substrate mix is added (prepared by the addition of a 5 mg tablet of orthophenylenediamine [Sigma Chemical Company] and 4 µ l 30% H_2O_2 to 25 ml of 0.01 M sodium citrate, pH 4.9), and the plates are shaken in the dark for 30 min at ambient temperature. Hydrochloric acid (50 µl; 4 M) is added to stop the reactions, and the absorbance of the wells is determined in a TITERTEK MULTISCAN NCC-340 plate reader (Flow Laboratories, McClean, VA) at 492 nm. Data are sent to a Zenith 80286 computer, and standard curves are plotted and levels determined using TITERSOFT software (Flow Laboratories). All values are determined in triplicate. This assay is specific for intact hCG, with less than 1% cross-reactivity with free beta-subunit, free alpha-subunit, and human luteinizing hormone (hLH).

Total hCG is quantitated in the same way except that a monoclonal anti-alpha-chain antibody (Unipath) 4 μg/ml in the microtiter plate coating step) is used in place of the B109 antibody, a change that renders the assay equally specific for intact and nicked hCG. This assay is specific for total hCG, with less than 0.1% cross-reactivity with hCG free beta-subunit, free alpha-subunit, or hLH.

EXAMPLE 2

A serum sample from a pregnant women at 26 weeks gestation is assayed for intact and total hCG according to the procedure in Example 1. The proportion of hCG that is in the intact form in the sample is found to be 10%. This value indicates that the woman is at increased risk for preterm delivery.

EXAMPLE 3

A cervicovaginal secretion sample from a pregnant women at 26 weeks gestation is assayed for intact and total hCG according to the procedure in Example 1. The proportion of hCG that is in the intact form in the sample is found to be 75%. This value indicates that the woman is not at increased risk for preterm delivery.

WHAT IS CLAIMED IS:

1. A method for determining an early indication of increased risk of preterm delivery comprising
   a. obtaining a body fluid sample from a pregnant patient after week 4 and before week 37 of pregnancy; and
   b. determining the proportion of total human chorionic gonadotropin that is in the intact form in the sample, a decreased proportion relative to that which is characteristic of pregnancies that proceed to term indicating an increased risk of preterm delivery.
2. The method of Claim 1 wherein the sample is a blood sample.
3. The method of Claim 2 wherein the sample is a serum sample.
4. The method of Claim 1 wherein the sample is a urine sample.
5. The method of Claim 1 wherein the proportion is determined by a non-quantitative assay.
6. The method of Claim 1 wherein the proportion is determined by a quantitative assay.
7. The method of Claim 6 wherein the level of total human chorionic gonadotropin and the level of intact human chorionic gonadotropin are quantitated using an immunoassay.
8. The method of Claim 7 wherein the immunoassay is a sandwich immunoassay.
9. The method of Claim 7 wherein the immunoassay is a competitive immunoassay.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/566, 33/567, 33/5[illegible] C[illegible]Q 1/00

US CL :436/501, 503, 510; 435/7.1, [illegible], 7.93, 7.94, 7.95

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/501, 503, 510; 435/7.1, 7.92, 7.93, 7.94, 7.95

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Biosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HENRY, "Clinical Diagnosis and Management by Laboratory Methods," published 1979 by W.B. Saunders Company (Philadelphia), see pages 680-692, see especially Figure 19 and pages 685-686.	1-9
Y	US, A, 4,310,455 (BAHL) 12 JANUARY 1982, see column 1, line 65 and column 12, line 42.	1-9
Y	US, A, 4,954,434 (MOROZ) 04 SEPTEMBER 1990, see column 5, line 31 and column 4, line 54.	1-9

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 June 1993	Date of mailing of the international search report 14 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DAVID R. PRESTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Endocrinology, Volume 129, No. 3, issued March 1991, L.A. Cole et al., "The hetrogeneity of human chorionic gonadotropin (hCG). III. The occurrence and biological and immunological activites of nicked hCG," pages 1559-1567, see especially page 1562, paragraph 1, right; page 1563, paragraph 2, left; page 1565, line 26, right; and page 1562, results section.	1-9

PCT WORLD INTELLECTUAL PROPERTY ORGANI
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/47, 14/775, C12N 5/08, G01N 33/68, 33/574, 33/92

A3

(11) International Publication Number: **WO 96/33214**

(43) International Publication Date: 24 October 1996 (24.10.96)

(21) International Application Number: PCT/US96/05441

(22) International Filing Date: 18 April 1996 (18.04.96)

(30) Priority Data:
08/423,409 18 April 1995 (18.04.95) US

(71) Applicant *(for all designated States except US)*: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, Oakland, CA 94612 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FISHER, Susan, J. [US/US]; 1347 4th Avenue, San Francisco, CA 94122 (US). GENBACEV, Olga [YU/US]; 1050 Crestview Drive #45G, Mountain View, CA 94040 (US). FOULK, Russell [US/US]; 740 Alta Vista, Pacifica, CA 94044 (US). CLAUSER, Karl, R. [US/US]; 3200 Treetops Circle, San Bruno, CA 94066 (US). BURLINGAME, Alama, L. [US/US]; 26 Alexander Avenue, Sausalito, CA 94965 (US).

(74) Agents: HUNTER, Tom et al.; Townsend and Townsend and Crew, Steuart Street Tower, One Market, San Francisco, CA 94105-1492 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
27 December 1996 (27.12.96)

(54) Title: ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

(57) Abstract

This invention is the discovery that hypoxic mammalian fetal trophoblast cells are a valid model of abnormal maternal plancental function and of other invasive cell types. The trophoblast cells differentially express a number of proteins when grown under hypoxic conditions. These proteins are useful for determining the health of the trophoblasts, the condition of a maternal placental interface and the invasive properties of metastatic and other invasive cell types. The hypoxic cytotrophoblast cells provide a convenient means to isolate these proteins and develope assay kits based upon the presence or absence of the proteins in a human test sample.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

INTERNATIONAL SEARCH REPORT

International application No /US 96/05441

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C07K14/775 C12N5/08 G01N33/68 G01N33/574 G01N33/92

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,5 141 849 (CHOU JANICE) 25 August 1992 see the whole document ---	1-3,7-13
X	DATABASE WPI Section Ch, Week 8207 Derwent Publications Ltd., London, GB; Class B04, AN 82-13463E XP002011721 & SU,A,827 030 (MOSC MED STOMATOLOG) , 7 May 1981 see abstract ---	1-4,7-13
X	WO,A,93 24838 (ADEZA BIOMEDICAL CORP) 9 December 1993 see the whole document --- -/--	1-4, 11-13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1996	23. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Chakravarty, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In[illegible] application No
PC[illegible] 96/05441

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 943 527 (PROTTER ANDREW A ET AL) 24 July 1990 see the whole document ---	1-4
X	US,A,5 196 523 (LEE A.S.) 23 March 1993 see the whole document ---	1-4
X,P	J. CLIN. INVEST, vol. 97, no. 2, 15 January 1996, NEW YORK, NY; US , pages 540-550, XP000579303 GENBACEV O ET AL.: "Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia" see the whole document -----	1-22

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ion on patent family members

...pplication No
.../US 96/05441

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-5141849	25-08-92	NONE		
WO-A-9324838	09-12-93	NONE		
US-A-4943527	24-07-90	AU-B-	6522486	24-04-87
		EP-A-	0239631	07-10-87
		JP-T-	63501764	21-07-88
		WO-A-	8702062	09-04-87
US-A-5196523	23-03-93	NONE		